The Toxicology of Nitric Oxide *In Vivo*

by

Aharon Gal

B.Sc., Emory University, Atlanta, Georgia
(1990)

SUBMITTED TO THE DEPARTMENT OF CHEMISTRY
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ABSTRACT

All multicellular organisms are at peril of developing cancer. Much effort has been invested in studying cancer development yet the underlying causes of the disease are still obscure. One hypothesis suggests that free radicals produced by the body as part of normal physiological functions cause damage to cellular molecules, particulary DNA. This slow accumulation of damage leads to mutations. Central to the free radical theory of cancer development is the observation that under conditions of chronic inflammation, macrophages and other inflammatory cells migrate to the inflammation site and release reactive free radicals. The accumulation of damage caused by these free radicals at the site of inflammation have been hypothesized to lead eventually to the development of common human malignancies such as viral hepatitis induced liver cancer and *Helicobacter pylori* induced stomach cancer.

Finding molecular evidence for this hypothesis has proved to be a daunting problem. Animals can not be dosed with radicals as is commonly done with chemical carcinogens and indirect methods of dosing need to be developed for these studies. Radicals are transitory species and therefore difficult to detect and quantify. The recently discovered free radical nitric oxide (NO•) is produced by macrophages during inflammation. NO• has been previously detected in several human malignancies. Studies of nitric oxide in cell culture and cell free systems have suggested that this radical may be an important mediator of macrophage-derived inflammatory damage. The objective of this thesis was to evaluate the damage caused by activated macrophages in an animal model of inflammation and assess the contribution of nitric oxide to macromolecular damage.

The first step in this work was to identify and characterize an animal model where high nitric oxide production can be induced. It was discovered that the injection of RcsX lymphoma cells into SJL mice leads to activation of host spleen and lymph node macrophages to produced nitric oxide. The second step of this work was to develop tools for the study parameters of cellular damage and apply them to this model. It was observed that production of nitric oxide led to cell death and damage to cellular proteins in NO• secreting macrophages and cells juxtaposed to them. The third part of this work evaluated the mutagenic potential of NO• in this model. A mutation detection system was incorporated into the SJL model and mutation frequencies were measured in mice induced to produced NO• and in controls. It was observed that nitric oxide production was mutagenic in our model.

In conclusion, this work established a model for the study of the macrophage-derived NO•, developed a set of tools to measure the toxicological effects of NO• and applied the tools to evaluate the effects on NO• in the model. The results obtained in this thesis demonstrate for the first time that free radicals can cause macromolecular damage and mutations *in vivo*.

Thesis Supervisor: Gerald N. Wogan
Title: Underwood-Prescott Professor of Chemistry and Toxicology

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Several scientists have made a substantial contribution to this work. The laboratory of Professor Jan Vijg in Beth Israel hospital in general and Michael E.T.I. Boerrigter and Martijn E.T. Dolle in particular have been extremely generous with their time and materials. I would also like to thank the numerous members of the Tannenbaum and Wogan groups who reviewed and corrected my thesis. Special thanks are due to my long time companions in the basement of building 26 who tolerated me throughout these years; in particular Zhifeng Zhang- who should be acknowledged both as a co-worker and as a personal friend, Laura J. Trudel- who stuck it out until we learned to live with each other, and Fayad Sheabar, my compatriot, who proved to me that Jews have a lot more in common with Arabs than with anybody else. I will miss you all.

A lot of people of conspired to keep me sane throughout the past six years. I would like to single out Dave Bem, Kurt R. Kendall, Can Ozbal and Olya Oliker for their cynicism, sarcasm and fondness of alcohol; they have all been well appreciated. The Rosenfeld tribe of Westport, Ct. has been my adoptive family far away from home. The wonderful friendships of Scott, Bevy, Alan, Rhona, Mitch, Donna and finally Robert A.H. have been a great source of support.

Snait Tamir deserves a separate acknowledgment. She was a wonderful friend and a wonderful guide through the most difficult parts of this thesis. I doubt very much that this work would have ever been accomplished without her.

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1. Background and literature review

The work described in this thesis incorporates issues from three different areas of research: nitric oxide toxicology, immunity and autoimmune disorders, and mutation research. In this chapter of the thesis pertinent literature of these three fields is reviewed and a background for the presentation of the project is constructed. The first section of the chapter briefly summarizes the field of nitric oxide chemistry and reviews the literature regarding the toxicology of nitric oxide. The second section of the chapter describes the SJL mouse, its predisposition to autoimmunity and the lymphoma model used to study nitric oxide toxicology. The third section describes the tools currently available for the study of *in vivo* mutagenesis followed by the details of the model used in our experiments.

Nitric Oxide

Nitric oxide production in human malignancies

The association of certain malignancies with chronic inflammation is largely an accepted hypothesis (1, 2), but a causal relationship has yet to be proven. Central to the concept that inflammation may play a role in the carcinogenic process is the finding that a chronic irritation of parenchymal tissue results in migration of inflammatory cells to the injured site where they release reactive free radicals hypothesized to facilitate carcinogenesis (3, 4). Recent data have shown that the free radical nitric oxide is present in inflammatory conditions associated with malignancies. Some of these preneoplastic conditions are reviewed below.

I) A model of viral infection: hepatitis B virus and liver cancer

Strong epidemiological evidence links hepatitis B virus (HBV) infection with hepatocellular carcinoma (HCC). Male HBV carriers, for example, have a 40% lifetime risk of HCC (5). The progression of HBV infection toward malignancy usually involves liver cirrhosis. There is some evidence that during liver cirrhosis in humans there is an increased plasma level of nitric oxide metabolites, nitrate and nitrosamino acids (2). Additional evidence comes from the woodchuck model of HBV. Nitric oxide production was elevated three fold (7.71 vs. 2.58 umol/day) in woodchucks chronically infected with the woodchuck hepatitis virus (6). Liu *et al.* reported that hepatocytes isolated from woodchuck hepatitis virus-infected animals were primed for nitric oxide production and secreted twice as much NO• after stimulation with lipopolysaccharide (7) as did uninfected woodchucks. Furthermore, woodchuck hepatitis virus surface antigen (WHsAg) stimulated hepatocytes in culture to produce nitric oxide (8).

II) A model of parasite infection: *Opisthorchis viverrini* and cholangiocarcinoma

The parasite *O. viverrini* is a liver fluke which infects approximately seven million people per year in northern Thailand (9) and is a major risk factor for cholangiocarcinoma with a risk odds ratio of 5.0 (10). The parasite is consumed in uncooked or undercooked fish and colonizes the intrahepatic bile ducts where it can live for many years if untreated (11). The bile duct inflammation which accompanies the infection is suspected of playing a role in the carcinogenic process. When cohorts of highly-infected, moderately-infected and

non-infected Thai villagers were compared for plasma, urine and saliva nitrate, the nitrate content increased with infection status. Furthermore, treatment with praziquantel, which kills the liver fluke, led to a substantial decrease in fluid nitrate content (9).

Additional support for the involvement of nitric oxide in *O. viverrini* infection comes from the hamster model of the disease. Ohshima *et al.* found that hamsters infected with *O. viverrini* excreted increased amounts of urinary nitrate and had a two fold higher nitric oxide synthase activity in their liver. Furthermore, nitric oxide synthase was immunohistochemically localized to the inflammatory regions surrounding the infected ducts (12).

III) A model of bacterial infection: *Helicobacter pylori* and stomach cancer

In 1980, gastric cancer was the most common neoplasm in the world (669,400 new cases per year) (13). Epidemiological and case control studies have shown that infection with *H. pylori* is a major risk factor for the disease (14, 15). *H. pylori* infection causes chronic gastritis which involves an increase in inflammatory cell infiltration into the stomach and formation of free radicals (reviewed by Lui *et al.* (5)). Recently, Correa and co-workers have localized iNOS, nitrotyrosine and apoptotic nuclei in stomachs of *H. pylori* infected individuals before and after bacterial eradication with antibiotic treatment. All three parameters positively correlated with infection status. These results constitute the strongest evidence reported to date for the presence of nitric oxide production in precarcinogenic states. In that paper the Correa group reported the first attempts to control NO•-induced damage by antioxidant treatment (16).

IV) A model of particle induced neoplasm: asbestos in lung cancer

Asbestos is a family of crystalline hydrated silicates which were commonly used in the construction industry. Occupational exposure to asbestos has been shown to be a major cause of mesothelioma, lung cancer, and is believed to be involved in cancers of the gastrointestinal track, larynx, kidney and several others (for review see Mossman and Gee (17)). Asbestos exposure usually occurs by inhalation followed by the deposition of the fibers in the lung. This leads to macrophage activation, toxicity, inflammation and the production of radicals (18, 19). Nitric oxide, in particular, was implicated in asbestos-induced inflammation by Thomas *et al.* who demonstrated that two types of asbestos (crocidolite and chrysolite) can activate rat alveolar macrophages to produce nitric oxide (20).

Synthesis, regulation, and reactivity of nitric oxide

Synthesis of nitric oxide

Nitric oxide is synthesized from L-arginine by a family of nitric oxide synthases (EC 1.14.13.39) (figure 1a-1). Three nitric oxide synthases have been discovered in mammalian cells and are generally referred to as NOS1, NOS2, and NOS3. NOS1 and NOS3 are constitutive enzymes regulated by allosteric effectors whose main function is to signal adjacent or nearby cells. The observed release of nitric oxide by cells expressing NOS1 and NOS3 is brief in duration and minute (nanomolar) in concentration. In contrast, NOS2 (inducible nitric oxide synthase, iNOS) is an inducible enzyme expressed most often in macrophages, whose activity cannot be allostericaly modulated. Activation of macrophages to express the inducible isoform leads to the continuous release of NO• over days or weeks at rates on the order of 5×10^3 to 5×10^4 molecules/cell-sec. Experiments in mice with different inherited inflammatory conditions and NOS knockout mice have shown unequivocally that the iNOS isoform is solely responsible for the role of nitric oxide in immunity and inflammation (summarized in (21)). Since it is this role of NO• that may lead to neoplastic progression, the following review will concentrate on iNOS.

The mouse inducible nitric oxide synthase is a cytoplasmic enzyme with a molecular weight of 130 kDa and 88% homology to the human isoform (reviewed in (22)). The enzyme functions as a dimer and catalyzes the conversion of L-arginine to L-citrulline and NO• consuming oxygen and five electron-reducing equivalents of NADPH in the process. iNOS is the only mammalian enzyme that combines a P-450 heme functional domain with a reductase domain in the same polypeptide and is believed to be related to bacterial monooxygenases (23). The enzyme requires 5,6,7,8-tetrahyrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and iron-protoporphyrin IX. Ca^{2+}/calmodulin is also required; however, it is tightly bound to the enzyme making the enzyme insensitive to Ca^{2+} concentration. The mechanism of iNOS catalysis is an active area of research. It is known that the first step of NO• synthesis involves the hydroxylation of arginine to N^G-hydroxy-L-arginine, and a complete mechanism of iNOS catalysis has been proposed (24) (for reviews on iNOS structure / function see (22-24)).

Regulation of nitric oxide synthesis

Induction of iNOS expression and consequent NO• production has been achieved

by a variety of environmental insults (e.g., viral infection, particles, parasites, hot beverages) and xenobiotic compounds (e.g., 5,6-dimethylxanthenone-4-acetic acid, picolinic acid, UV light, ozone, bacterial polysaccharides) in cultured cells (2, 25). Several cytokines have been implicated in iNOS induction, most notably γ-interferon (26). Researchers have found that maximal NO• production is achieved in macrophages when γ-INF synergizes with LPS, IL-1ß, TNFα or T cell membranes (26-31). On the other hand, several molecules down-regulate iNOS expression in macrophages including TGFß, which destabilizes iNOS mRNA (32); glucocorticoids, which interfere with NF-κB regulation of iNOS transcription (33); progesterone (34); excess iron (35); and nitric oxide itself (36).

Many of these regulatory molecules have been shown to act at the transcriptional level. Despite the fact that the iNOS promoter has been only partially characterized and contains many poorly understood regulatory elements (25), three separate promoter elements have been defined. The first is a γ-INF response element spanning positions -913 to -1029, which is sufficient for induction of the transcription of a reporter gene. The second is an LPS response element located in positions -48 to -209, which cannot function independently, but synergizes with γ-INF to result in a ten-fold higher expression of iNOS (37). Both of these regions also contain NF-κB response elements. NF-κB has been shown to mediate the regulatory activity of glucocorticoids and several cytokines on iNOS expression (25, 33). A third response element regulated by hypoxia and picolinic acid resides in positions -227 to -209 and potentiates the activity of γ-INF in an LPS-independent fashion (38).

Nitric oxide reactivity

Nitric oxide exerts a number of diverse effects via redox and additive chemistry; by interacting with catalytic metal centers (e.g., hemes and Fe-S) and thiol groups (RS-NO•); and by reacting with other free radicals (e.g., O_2^{-}•) to form additional reactive species (39-42). The binding of NO• to metal centers has been shown to regulate the function of many enzymes including guanylate cyclase, aconitase, cyclooxygenase, ribonucleotide reductase, and cytochrome P450 mixed function oxygenases. The binding of NO• to thiols has been demonstrated in glutathione, albumin, tissue plasminogen activator and hemoglobin. The

reaction of NO• with $O_2^{-\bullet}$ leads to the formation of additional reactive molecules and is at least partially responsible for the bactericidal and tumoricidal activities of macrophages which will be discussed later.

Schmidt and Walter (39) have observed that the ability of nitric oxide to interact with many targets, which makes it so effective in signalling and fighting invasion, is also responsible for the substantial damage caused by NO• misregulation. For example, the production of NO• as part of the immune response of bacterially infected mice leads to activation of guanylate cyclase in endothelial cells, resulting in vasodilation and fatal septic shock (43). Additionally, the ability of NO• to inactivate ribonucleotide reductase or deplete glutathione may be responsible for its ability to induce cytostasis and neuronal cell death (39).

Although much has been learned about nitric oxide, the field is still struggling with some basic relevant biochemical concepts including the structure/function of iNOS, its mode of regulation, and the molecular targets of NO•. The most striking feature of this newly emerging picture is its complexity. As yet it is too early to decipher the regulatory aspects of nitric oxide production and their relevance to carcinogenesis. The work described here concentrates mainly on classical toxicological parameters of overexpressed nitric oxide: macromolecular damage; cell death; and mutations.

The chemistry and toxicology of nitric oxide

Metabolism of nitric oxide

Once produced, macrophage-derived nitric oxide can follow one of three pathways: it may diffuse out of the cell; autoxidize to $NO_2^{\bullet}$; or react with superoxide to form peroxynitrite. Diffusion of NO• into the extracellular environment and adjacent cells is possible because the cell membrane permeability of nitric oxide is similar to that of oxygen. Inside or outside the generator cell, NO• may transiently interact with metals or thiols, functioning as a signalling molecule or allosteric regulator, or it may undergo additional metabolism as described below.

Both autoxidation and peroxynitrite formation are promutagenic (figure 1a-2). Autoxidation of NO• eventually leads to the formation of N_2O_3, a powerful electrophilic nitrosating agent (figure 1a-3). Formation of N_2O_3 is important because it leads to deamination of DNA bases and formation of mutagenic N-nitrosamines. Nitrosation of

primary amines on DNA bases results in formation of diazonium ions which are subsequently hydrolyzed to alcohols leading to a variety of promutagenic lesions. Deamination of guanine to xanthine, for example, may result in miscoding or in depurination to form abasic sites in DNA and consequent single strand breaks or misrepair. Deamination of 5-methylcytosine would result in a G•C→A•T transition (figure 1a-4). Alternatively, reaction of the aryl diazonium ion of a nitrosated base with a nucleophilic site on an adjacent macromolecule could lead to crosslinking with other nucleic acids or with proteins (21). The other potential products of N_2O_3 formation, the N-nitrosamines, are well-known chemical carcinogens that are metabolized to strong alkylating electrophiles that react with DNA at several nucleophilic sites. This has been demonstrated both in cell culture and in animals with chemically synthesized and endogenously-formed N-nitroso compounds (figure 1a-5) (44).

Reaction of nitric oxide with superoxide is likely to dominate the fate of NO• *in vivo* because the kinetics are diffusion-limited. The product of this reaction, peroxynitrite (ONO_2^-), is a potent one-electron and two-electron oxidizing agent capable of destroying glutathione and ascorbic acid, and damaging DNA both by attacking the deoxyribose backbone and by directly oxidizing and nitrating purines and pyrimidines (figure 1a-6, 7)

Activated macrophages have been calculated to produce superoxide at about half the rate of nitric oxide (45) and may therefore be a major target of peroxynitrite themselves, a phenomenon which may have a role in fighting bacterial infection. However, peroxynitrite may form in any cell containing superoxide if that cell is close to an NO• producing macrophage (21). Strong evidence for the formation of peroxynitrite *in vivo* comes from animal experiments where tissue damage induced by immune reactions and reperfusion injury could be attenuated either by inhibition of NOS or by reduction of $O_2^{\bullet-}$ by superoxide dismutase (46, 47).

Direct evidence for DNA damage by NO•

A simple and elegant experiment that demonstrated the ability of nitric oxide to damage DNA was recently performed by Tamir *et al.* (48). In this experiment, the beta-galactosidase activity of a lacZ coding CMV plasmid was measured after exposure to activated macrophages under a variety of conditions. Macrophage derived NO• inhibited lacZ expression after treatment of the plasmid in solution, in macrophages, and in a host cell line.

DNA damage induced by macrophages on LacZ plasmid (48)

	% ß-galactosidase activity
[1]Macrophages + bare plasmid	100 ± 11
Macrophages + LPS + INFγ + bare plasmid	20 ± 4
Macrophages + LPS + INF–γ+ NMA + bare plasmid	57 ± 6
[2]Macrophages carrying plasmid	100 ± 6
Macrophages carrying plasmid + LPS + INF–γ	40 ± 2
Macrophages carrying plasmid + LPS + INF-γ + NMA	81 ± 2
[3]Macrophages + CHO cells carrying plasmid	62 ± 13
Macrophages + CHO cells carrying plasmid + LPS + INF-γ	18 ± 5
Macrophages + CHO cells carrying plasmid + LPS + INF-γ + NMA	100 ± 23

[1]Bare CMV plasmid was added to the media of stimulated and unstimulated macrophages for 6 h. The plasmid was then rescued and transfected into CHO cells and ß-gal activity was tested after 12 h.
[2]Macrophages were first transfected with the CMV plasmid and then were stimulated for NO• production. After 12 h cells were harvested and ß-gal activity was tested.
[3]CHO-AA8 cells were first transfected with the CMV plasmid and then were co-cultured (using inserts) with stimulated and unstimulated macrophages. After 12 h CHO cells were harvested and ß-gal activity was tested. The control was CHO cells carrying the plasmid but incubated in the absence of macrophages.

Several groups have characterized the DNA damage and mutations induced by nitric oxide and its reactive metabolites under a variety of experimental conditions and their results are presented in the table below. The results are consistent with the damage spectrum suggested by nitric oxide chemistry. Evidence has been found for deamination, oxidation and (probably peroxynitrite-dependent) strand breaks. Base pair substitutions consistent with deamination and oxidation have been reported for treatment with NO• and peroxynitrite, respectively. All mutation experiments reported thus far utilized systems unable to detect deletions larger than 100 bp. Furthermore, it should be noted that in most experiments the target was exposed to physiologically irrelevant doses of NO• and $ONOO^-$.

Experiments where targets were exposed to activated macrophages in cell culture have been performed in this lab with encouraging results. However, in cell culture, target cells are exposed to accumulating amounts of additional substances (cytokines, nitrate) that are cleared by the bloodstream in living organisms. Therefore, there is a need for a study of the DNA damage and mutagenicity of macrophage-derived nitric oxide *in vivo* . To date, no such study has been published.

DNA damage and mutations observed after nitric oxide and peroxynitrite treatment

Compound, delivery method and target	Observations	Reference
NO• gas on nucleosides and *S. typhimurium*	Deamination products *in vitro* and C→T in bacteria (reversion mutation: CCC target sequence).	(49)
NO• gas on TK6 cells	DNA deamination (xanthine, hypoxanthine), DNA strand breaks, hprt mutagenicity.	(50)
NO• gas on pSP189 (naked DNA)	15 and 44 fold increase in mutant frequency when plasmid was replicated in bacteria and human cells, respectively. Dominant mutations AT→GC and GC→AT.	(51)
NO• donor drugs on pSP189 (naked DNA)	7 and 14 fold increase in mutant frequency (DEA/NO and SPER/NO respectively). Dominant mutation: GC→AT.	(52)
NO• donor drugs with peroxynitrite on naked DNA	Formation of 8-oxoG, peroxynitrite mediated cleavage of DNA.	(53)

NO• donor drugs, iNOS transfection on BEAS-2B cells	No mutation in hprt or exon 7 of p53.	(54)
Activated cultured macrophages (Raw 264.7)	DNA deamination (xanthine) and oxidation {5-(hydroxymethyl)uracil}, FapyG and 8-oxoG.	(55)
Peroxynitrite on pSP189 (naked DNA)	Visible plasmid linearization on agarose gel from 1 mM $ONOO^-$ and up. 21 and 9 fold increase in mutant frequency when plasmid was replicated in bacteria and human cells respectively. Dominant mutations GC→TA and GC→CG.	(56)
Activated cultured macrophages	Formation of double strand breaks (correlated specifically with peroxynitrite formation).	(57)
NO• gas, peroxynitrite on CHO-AA8 cells and CHO-EM9 (SSB repair deficient)	Immediate formation of AP sites followed by single strand breaks (SSB) after 24 hours. $ONOO^-$ induced immediate formation of single strand breaks. In CHO-EM9 cells double strand breaks were observed after 24 hours for both NO• and $ONOO^-$.	(48)

Cytotoxic effects of nitric oxide

When challenged by lymphokines or bacterial antigens, macrophages respond in a sequence of defined steps culminating in the release of factors known to induce the death of affected cells including H_2O_2, TNFα, IL- Iß and NO• (44, 58-60). NO• is believed to be the dominant mediator of this macrophage-induced cytotoxicity (57, 61). NO• can kill cells through autoxidation to N_2O_3, reaction with $O_2^{\bullet-}$ to form peroxynitrite, and interaction with transition metals, notably iron centers. Results published thus far suggest that nitric oxide causes cell death via DNA damage-induced apoptosis. Evidence for NO• dependent programmed cell death had been found in macrophages (62-64), thymocytes (65),

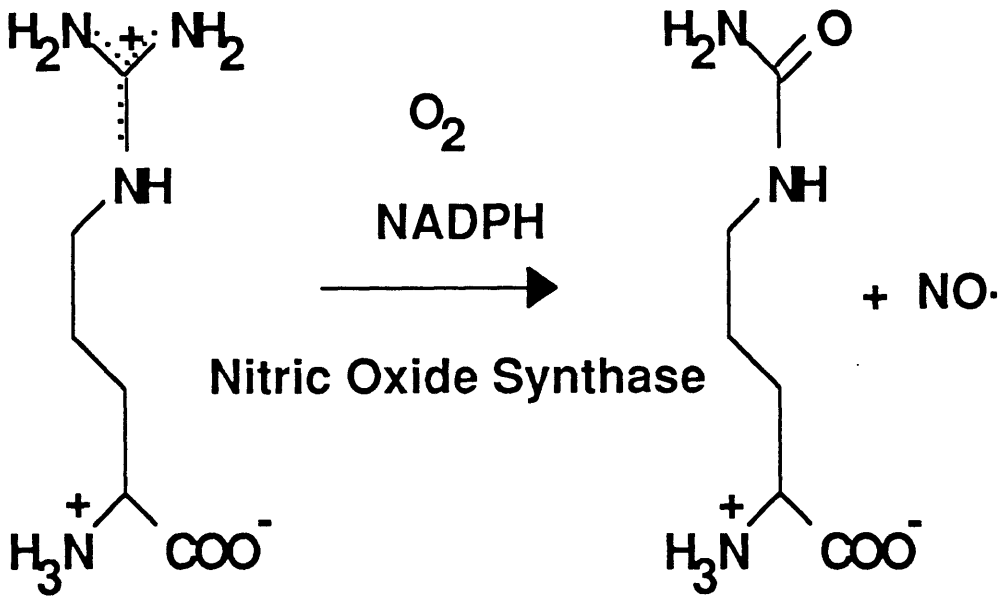
pancreatic cells (30, 66) and smooth muscle cells (67).

Several reports regarding the mechanism of NO•-induced apoptosis have been published. Luperchio *et al.* (68) observed that NO•-induced glutathione depletion is toxic in TK6 cells. Brune and co-workers have observed that exposure to nitric oxide led to accumulation of p53 prior to apoptosis in RAW 264.7 macrophages, pancreatic RINm5F cells and thymocytes (65, 69). The same group also observed that up-regulation of protein kinase A and protein kinase C prevented apoptosis induced by NO• donor drugs while down-regulation of protein kinase C potentiated the process (70).

A suggested mechanism for nitric oxide-induced cell death has recently been put forward. Poly(adenosine 5'-diphosphoribose) polymerase (PARP) is a nuclear enzyme which, when activated by DNA strand breaks, adds up to 100 adenosine 5'-diphosphoribose (ADP-ribose) units to nuclear proteins using ß-nicotinamide and ATP in the process. Excessive activation of PARP may lead to depletion of ß-nicotinamide-ADP and ATP. Snyder and co-workers observed that PARP is activated by NO• and that addition of PARP inhibitors prevents NO• induced neurotoxicity (71). Heller *et al.* further demonstrated that NO•-induced toxicity in pancreatic islet cells is suppressed in mice having an inactive PARP gene (72). Further evidence in support of the connection between strand breaks, PARP and apoptosis was provided by Szabo and coworkers, who demonstrated that in macrophages and smooth muscle cells, macrophage-derived peroxynitrite induced DNA strand breaks which led to activation of PARP, depletion of intracellular NAD^+ and ATP, decrease in mitochondrial respiration and cell death (57, 67).

In summary, considerable progress has been made in the study of nitric oxide toxicology *in vivo* and *in vitro*. *In vivo*, the presence of nitric oxide has been demonstrated in models of inflammation induced carcinogenesis. *In vitro*, the chemical reactivity and the potential molecular targets of nitric oxide have been identified. The relevance of the damage markers identified *in vitro* to mammalian physiology is yet to be established.

Synthesis of nitric oxide from arginine



L-arginine L-citrulline

Figure 1a-1: Synthesis of nitric oxide. Nitric oxide synthases catalyze the conversion of L-arginine to nitric oxide and L-citrulline consuming one molecule of oxygen and five-electron reducing equivalents of NADPH in the process.

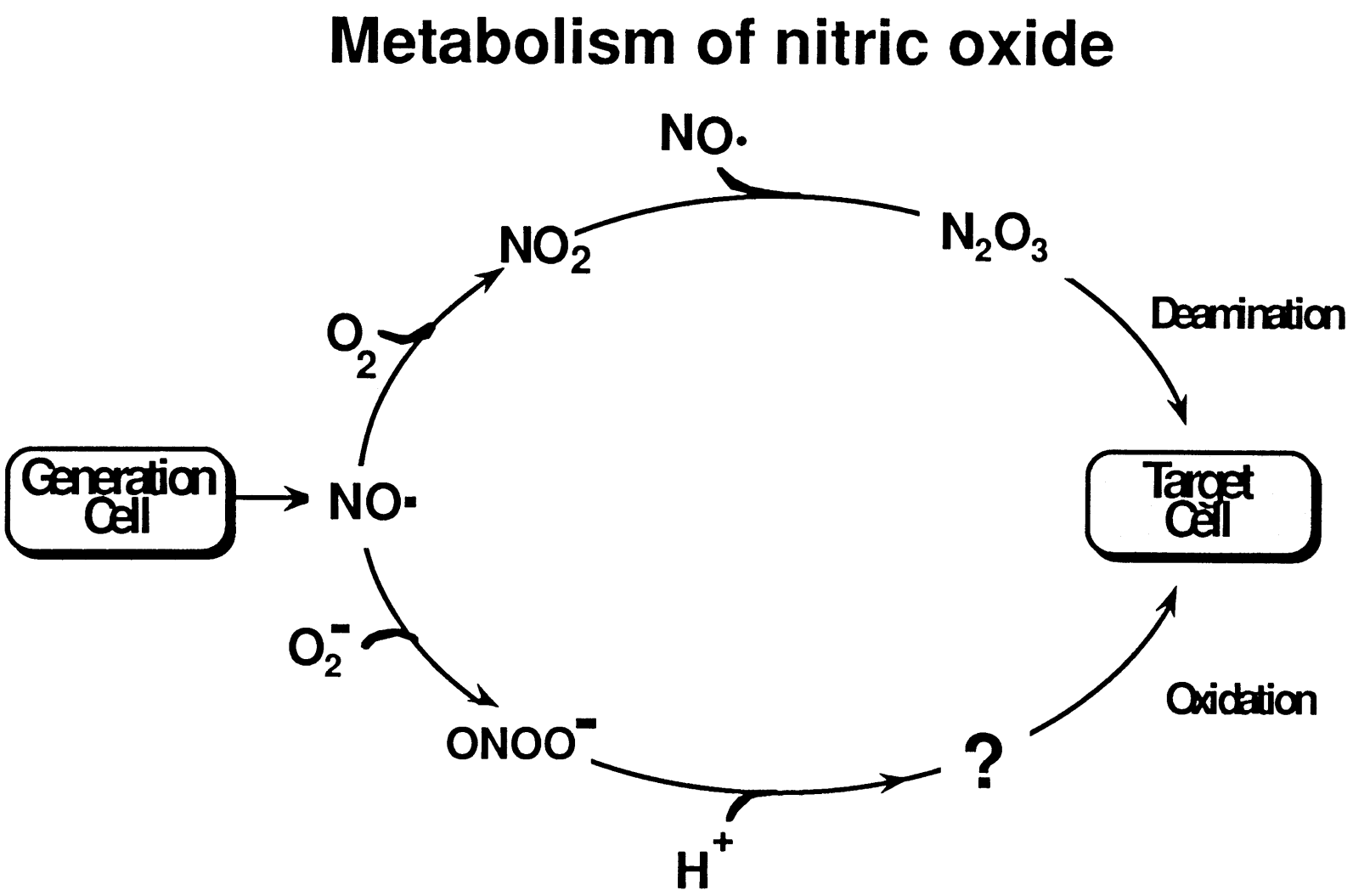


Fig 1a-2: Metabolism of nitric oxide. Nitric oxide may react with oxygen to form N_2O_3 or with superoxide to form peroxynitrite. Both metabolic pathways have been demonstrated to lead to DNA damage.

Autoxidation of nitric oxide

$$2NO\cdot + O_2 \longrightarrow 2NO_2$$

$$NO\cdot + NO_2 \longrightarrow N_2O_3$$

$$N_2O_3 + H_2O \longrightarrow 2H^+ + 2\,NO_2^-$$

$$N_2O_3 + \text{amine} \longrightarrow \textit{N}\text{-nitrosamine} + NO_2^-$$

Figure 1a-3: Deamination chemistry of nitric oxide. In an aerobic environment, nitric oxide autoxidizes to form N_2O_3. The first step of the reaction is second order with respect to nitric oxide and the reaction is slow at low nitric oxide concentrations.

Mutagenicity of deaminated bases

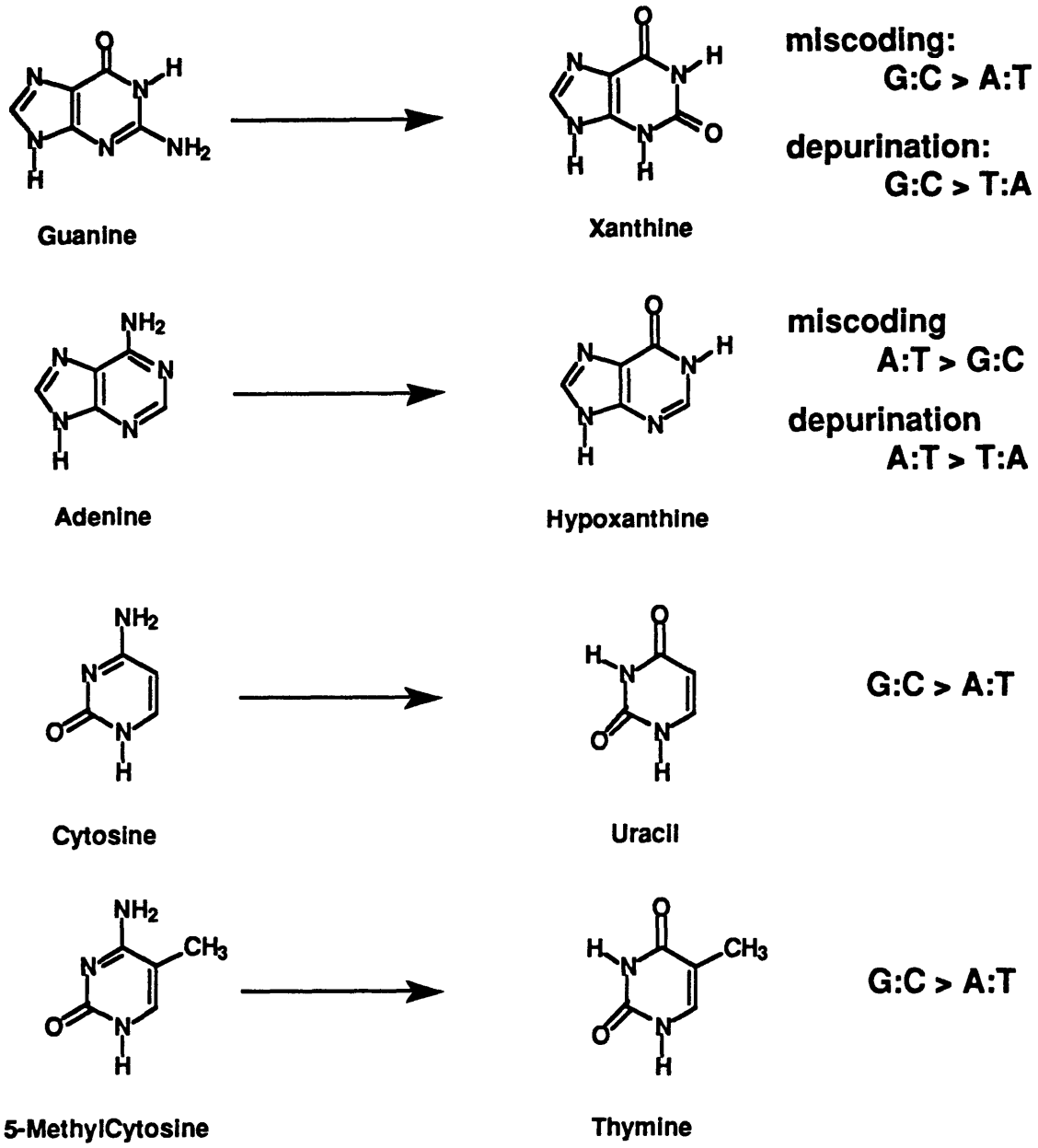


Figure 1a-4: Deamination and potential mutations. Autoxidation of nitric oxide leads to the formation of N_2O_3. Reaction of N_2O_3 with primary amines on DNA bases results in formation of diazonium ions and subsequent deamination.

DNA deamination and alkylation by N_2O_3

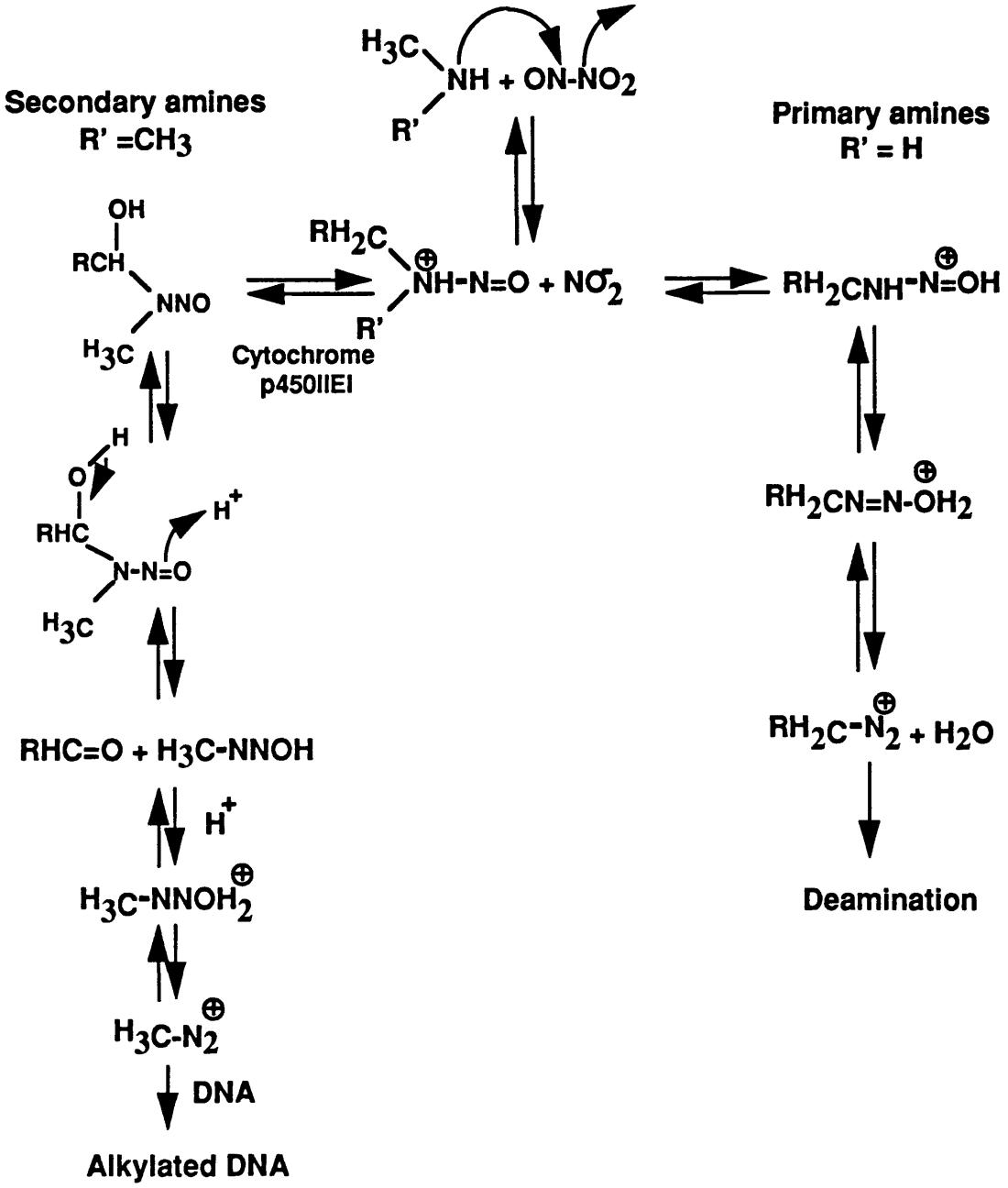


Figure 1a-5: Formation of N_2O_3, alkylation, and deamination of DNA by N_2O_3. Autoxidation of nitric oxide leads to the formation of N_2O_3. Nitrosation of primary amines on DNA bases results in deamination and nitrosation of secondary amines may result in the formation of alkylating scecies.

Oxidation chemistry of nitric oxide

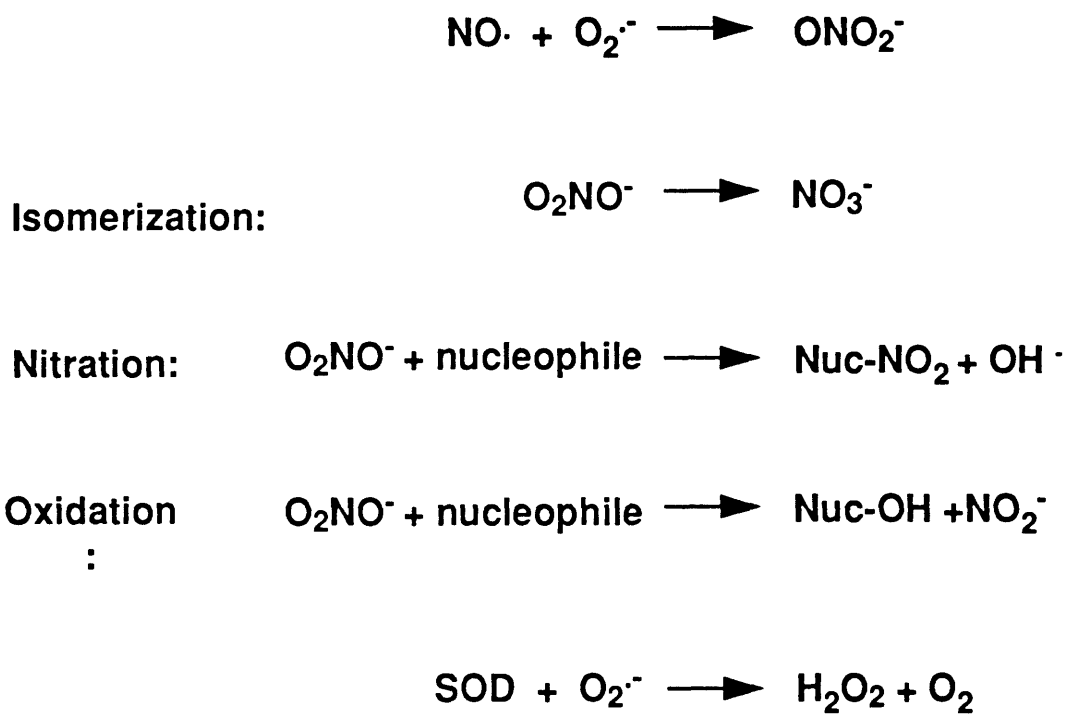
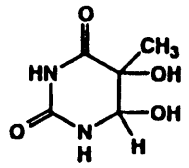
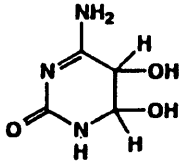


Figure 1a-6: Oxidation chemistry of nitric oxide. Nitric oxide reacts rapidly with superoxide to form peroxynitrite. Peroxynitrite may, in turn, isomerize to nitrate, nitrate target molecules or oxidize them. The oxidation pathway is in competition with the removal of superoxide by superoxide dismutase (SOD).

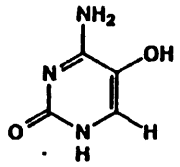
Oxidized DNA bases



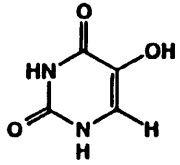
Thymine glycol (cis- and trans-)



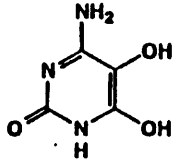
Cytosine glycol



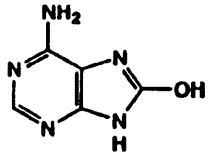
5-Hydroxycytosine



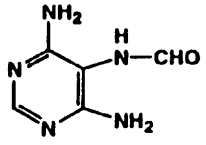
5-Hydroxyuracil



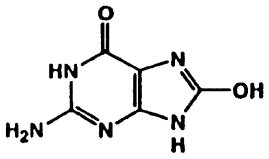
5,6-Dihydroxy cytosine



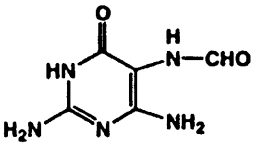
8-Hydroxyadenine



4,6-Diaminop-5-formamidopyrimidine



8-hydroxyguanine



2,6-diamino-4-hydroxy-5-formamidopyrimidine

Figure 1a-7: Some examples of oxidized DNA bases. Under physiological conditions peroxynitrite is a potent oxidizing agent which may lead to the formation of any or all the oxidized bases mentioned above.

The SJL mouse

Immunodeficiencies of the SJL mouse strain

The SJL mouse strain was originally derived from swiss Webster mice by Edwin D. Murphy (73). SJL mice exhibit multiple immunological disorders, including paraproteinemia (74), myositis (75), and slow maturation of the ability to mount a delayed-type hypersensitivity response (76). Self tolerance can easily be broken in SJL mice (77, 78) and the strain is used as a model for autoimmune encephalomyelitis (79), viral encephalomyelitis (80), experimental myositis (81), muscle regeneration (82), and experimental colitis (83).

Several research groups have attempted to define the underlying molecular basis for the immunological defects in SJL mice. Behlke *et al.* have shown that the genetic locus of the Vß region of the T cell receptor is 70% deleted in SJL mice (84) resulting in a limited repertoire of T cell receptors. Since the T cell receptor plays a key role in cell-mediated immunity, this aspect of the SJL immune system is likely to be deficient. In a separate study, SJL mice have been shown to have few suppressor T cells and are, therefore, incapable of moderating immune responses (85, 86). SJL mice have almost no natural killer cells due to suppression of natural killer cell maturation by the thymus during the first few weeks of life (87, 88). A deficiency in the macrophage compartment has recently been shown (by cell transfer) to be responsible for the inability of young SJL male mice to mount a delayed type hypersensitivity response against soluble antigens (89). Recently, several groups have observed that cross-regulation of the immune system by lymphokine secretion is deficient in SJL mice. Maley *et al.* demonstrated that the skeletal muscles of SJL mice are hyper-responsive to basic fibroblast growth factor (82); Yoshimoto *et al.* showed that SJL mice cannot produce interleukine-4 in response to immunization with anti-IgD antibody (90); and Vidard *et al.* demonstrated that SJL mice have a cytokine

profile different from other mice after immunization with ovalbumin (91).

The molecular basis for spontaneous lymphoma development in SJL mice

In his original paper, Murphy reported that SJL mice have a high frequency of spontaneous lymphatic tumors he termed 'reticulum cell sarcomas' (RCS) starting at one year of age (73). The high incidence of the disease and the histological appearance suggested the use of this mouse strain as an experimental model for Hodgkin's disease (92). After much debate, it is now agreed that these spontaneous RCS tumors belong to the B cell lineage and possess rearranged immunoglobulin genes (93, 94). When tumor lines were derived by successive transplantation in young SJL mice, it became apparent that they had some unusual properties (reviewed by Ponzio *et al.* (95)). All RCS tumor cells presented a surface antigen capable of stimulating a subset of T cells of healthy SJL mice. All T cells that grew in response to the SJL lymphoma were later found to express the same variable chain of the T cell receptor, Vß-16 (96). When stimulated, the Vß-16+ T cells secreted a mixture of cytokines *in vivo* and *in vitro*. The tumor cells, in turn, could not grow without these cytokines, most notably IL-5 and γ-interferon (97-99). In addition to T cells, growth of the tumors in host animals appears to require the presence of macrophages. When host SJL mice were pre-treated with macrophage inhibiting substances (trypan blue or carrageenan), tumor growth was retarded. Irradiated SJL mice could not support tumor growth after being repopulated with peripheral T cells alone unless macrophages were co-transferred with the T cells (100).

The molecular basis for the ability of the RCS tumors to stimulate the Vß 16+ T cells was determined by Tsiagbe *et al.*, who demonstrated that a superantigen derived from the mouse mammary tumor virus (MMTV) is expressed on the surface of SJL pre B cell lymphomas and is required for their ability to stimulate host T cells (96). Superantigens are

molecules that bind and stimulate families of T cells expressing a particular T cell receptor variable ß-domain. Superantigens interact with a higher fraction of T cells than conventional antigens, resulting in a potent inflammatory immune response (for a review of superantigens see Herman *et al.* (101)). The MMTV virus is a retrovirus first isolated on the basis of its ability to cause mammary tumors in mice (102). In 1991, it was observed that MMTV is capable of infecting B cells and that proviral genes are expressed in cells of the B cell lineage starting as early as the pro-B cell stage (103). Concurrently, another group proved that the long terminal repeat of MMTV codes for a superantigen (104). The MMTV superantigen induces an immune stimulation which supports the amplification of the virus (105). It is hypothesized that the SJL mouse strain was exposed to the MMTV virus and incorporated a truncated version of a Vß16-specific superantigen into its genome. When exposed to superantigens, most mice strains fight the aberrant immune stimulation by deleting the superantigen-responsive cells (106). SJL mice, in contrast, do not delete the Vß 16^+ T cells.

This evidence suggests a possible mechanism for the progression of the disease. In SJL mice, as B cells follow their differentiation program, they begin to express the superantigen on their surface. The host Vß 16^+ T cells respond vigorously, secrete cytokines and stimulates the pre-B cells to grow, resulting in a positive stimulation cycle. While in other strains this feed-forward mechanism would have been ended by the deletion of the Vß 16^+ T cells, in SJL mice the cycle of stimulation continues unchecked, resulting in selection of increasingly aggressive cells and culminating in the formation of a tumor.

Nitric oxide production in spontaneous myositis in SJL mice

We have been interested in using the SJL mouse as an *in vivo* model for the study of nitric oxide toxicology. It has recently been observed that SJL mice spontaneously

develop autoimmune myositis (75) in addition to the lymphoma discussed above. Since macrophages have been shown to comprise more than 80% of the infiltrating cell population in experimental autoimmune myositis (81) and suggested to play a role in the lymphoma (100), we have investigated the production of nitric oxide during the development of spontaneous myositis in aging SJL mice (107). Cohorts of SJL and BALB/c mice were followed for 41 weeks. Elevated NO• production, measured by urinary nitrate excretion, by aging SJL mice as compared to BALB/c control mice was observed. The presence of inducible nitric oxide synthase was detected immunohistochemically in infiltrating macrophages in old SJL mice but not in BALB/c controls. The severity of myositis in individual mice, scored histopathologically, correlated with urinary nitrate excretion (figure 1b-1). Furthermore, administration of NMA to mice in the early stages of myositis development significantly reduced the progression of the disease. These results proved that nitric oxide was indeed produced by activated macrophages in the inflamed muscle of the SJL mouse and was significant for the progression of the disease.

Unfortunately, the spontaneous myositis model was inappropriate for the objective of this work; the analysis of NO• induced macromolecular damage. The model was exceedingly slow. Mice would have had to be followed for an entire year for each experiment. In addition, there was substantial individual variation between mice (figure 1b-2). The age of onset of clinical myositis and rise of urinary nitrate levels tended to vary dramatically between mice. For the purpose of this work, a model with a faster and more uniform response was needed.

The RcsX cell line

The tendency of SJL mice to develop autoimmune disease, the stimulation of the host immune system in the lymphoma model, the dependence of transplanted lymphoma cells on macrophages, and the production of gamma interferon by cultures of lymphoma

cells suggested the possibility that transplantation of a lymphoma cell line into SJL mice might induce NO• production by host macrophages.

To test this hypothesis, we decided to use the transplantable tumor cell line RcsX (97). This cell line was chosen because it was better characterized than other SJL transplantable tumor lines. The RcsX tumor line was derived by successive transplantation of a lymphoma in young SJL mice (94). RcsX lymphoma cells were found to express MHC I-A^s, antigenic B cell markers (98) and the MMTV LTR-derived superantigen (96). The RcsX cell line was shown to be a fully transformed cell line, capable of forming colonies in soft agar (98) and stimulating T cell growth *in vitro* (95). Intraperitoneal injection of 10^7 RcsX cells into SJL mice leads to a rapid expansion of tumor and host T cells in the lymph nodes, spleen and liver of the host leading to morbidity 15 days later (95). This cell line has been used in all the work described in this thesis.

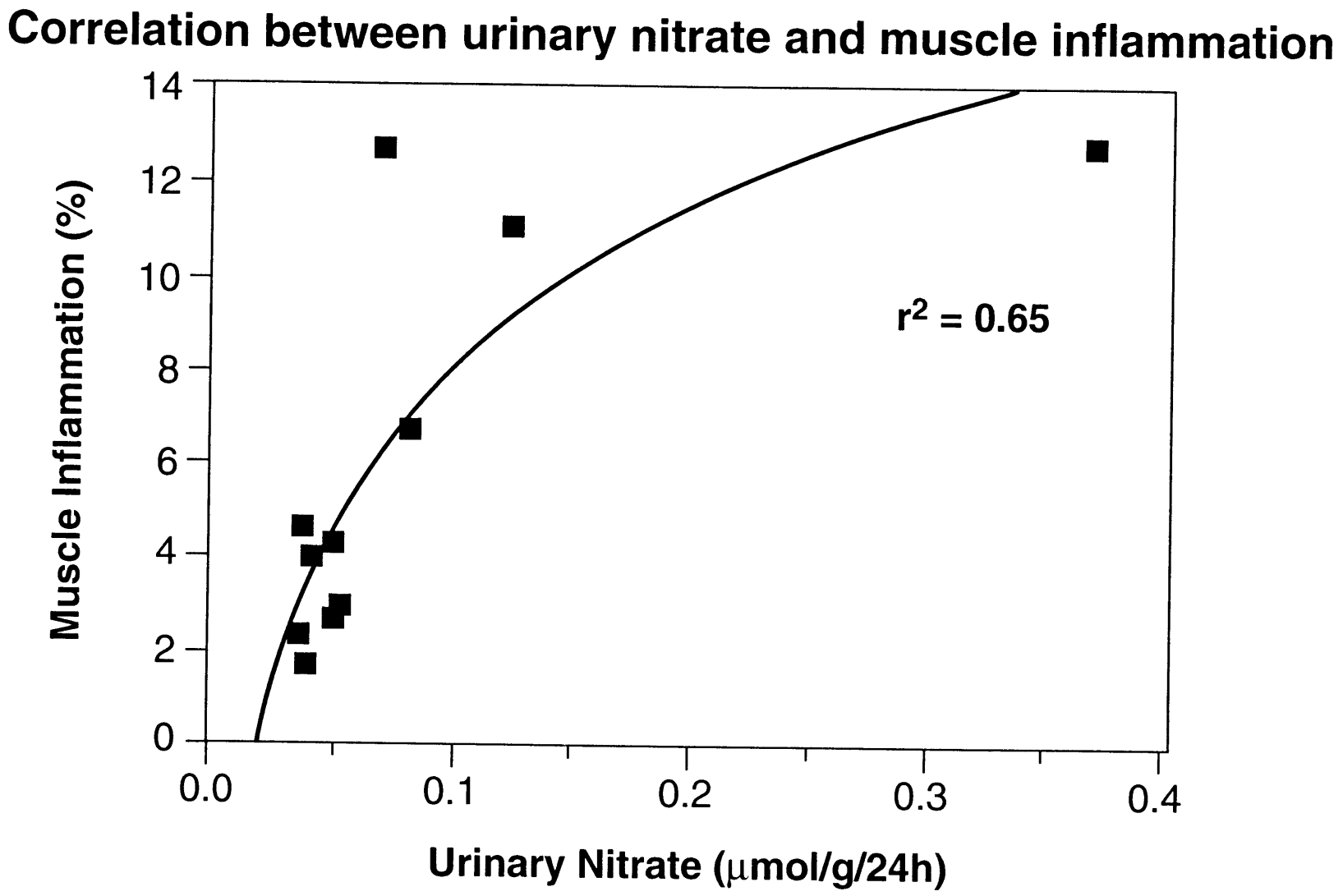


Figure 1b-1: Nitric oxide production correlates with muscle inflammation in aging SJL mice. Urinary excretion of nitrate, the end metaboblite of nitric oxide, positively correlated with pathologically scored inflammation in voluntary muscles in one year old SJL mice (adapted from Tamir, *et al.* PNAS 95: 1234 - 1245).

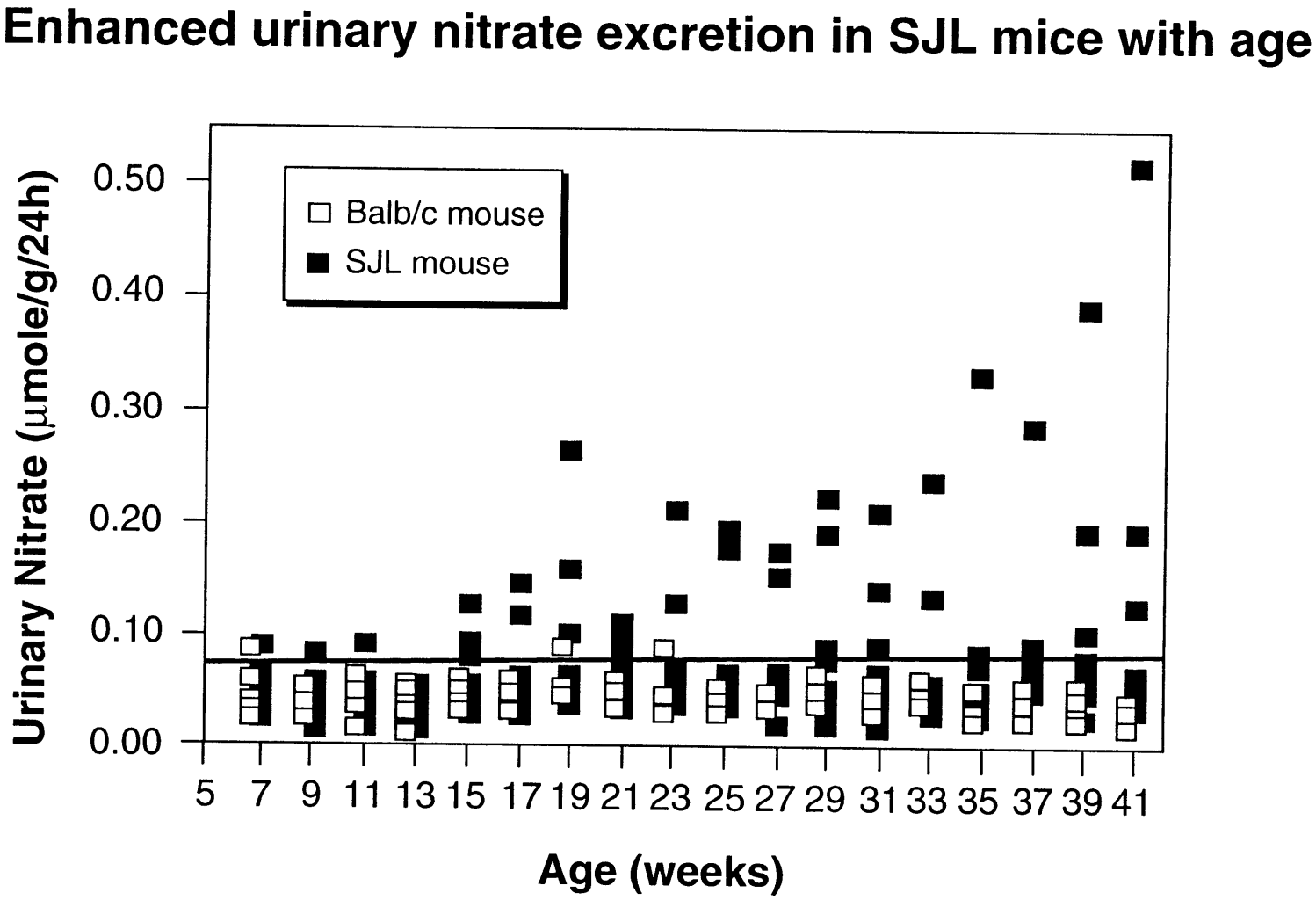


Figure 1b-2: Urinary nitrate excretion by aging SJL mice. SJL mice develop myositis during the first year of life. Nitric oxide is produced as part of the disease progression and is ultimately excreted as urinary nitrate. The progression of the disease varies greatly between individual mice. (adapted from Tamir, *et al*. PNAS 95: 1234 - 1245)

Analysis of mutations induced *in vivo*

A causal link between mutations in key genes and cancer has been convincingly established in the last ten years (108). Proponents of the free radical theory of cancer development have suggested that endogenously produced reactive species are responsible for a steady stream of random mutations that eventually leads to transformation and tumorigenesis (3). It was yet to be demonstrated, that free radicals are capable of inducing mutations *in vivo*. In the past several years, experimental systems designed to evaluate *in vivo* mutagenicity have been developed. This section discusses different mouse mutant analysis systems, their advantages, disadvantages, and relevant results.

Mutation analysis systems in mice

There are five commonly used *in vivo* mutation analysis models, each named after the mouse genetic sequences used as target. Two systems use endogenous genes: hypoxanthine-guanine-phosphoribosyl-transferase (hprt) and dlb-1^b; and three use transgenes: the lac-Z phage system (ΦlacZ, mutamouse™), the lac-I phage system (ΦlacI, Big Blue™) and the lacZ-pUR288 plasmid system (pUR288, Hugo™). The endogenous systems have two advantages over the transgenic systems. First, mutations occur in unperturbed genes in their native chromosomal conformation. This makes endogenous gene systems a better model for oncogenes and tumor suppressor genes than the long stretches of transgenic DNA randomly integrated into the mouse's genome. Second, mutations detected in endogenous genes have all been induced in host cells and transmitted during cell division. In contrast, mutations in transgenes are scored in bacterial hosts and may or may not have been established in the host cells. However, the endogenous mutation models also have two severe shortcomings. First, they are limited to a single organ and cell type (dlb-1^b to intestinal epithelial cells and hprt to splenic T cells). Second, the assays are difficult and cumbersome to perform. The transgenic systems were designed to overcome these two basic problems. They can be applied to all organs, they are easy to perform, and both mutation frequency and spectrum can be determined with relative ease.

The dlb-1^b model is based on an endogenous gene product that is a surface protein present in the mouse intestinal villi. The protein is responsible for binding a lectin derived from *Dolichos bifloruse*. Because the intestinal epithelium is completely renewed every

four or five days mutations in the dlb-1^b gene in intestinal stem cells in dlb-1^a/dlb-1^b heterozygous mice lead to the total loss of the lectin binding site in an intestinal villus within one week. Dlb-1^b mutations can be visualized by staining intestinal mounts with *D. bifloruse* lectin agglutinin-peroxidase conjugate and an appropriate chromogen. While this system is relatively easy to use, the dlb-1^b gene has yet to be cloned and the system has had limited use (109).

The hprt gene encodes for the hypoxanthine-guanine-phosphoribsyl-transferase enzyme which is part of the salvage pathway of nucleoside synthesis. Inactivation of the hprt gene product confers resistance to purine analogs, such as 6-thioguanine. Since the hprt gene is X-linked, a mutation in the active allele is sufficient for phenotypic selection. The selection is performed on splenic T cells grown *in vitro* in the presence of T cell mitogens and 6-thioguanine. DNA can be amplified by polymerase chain reaction from 6-thioguanine resistant colonies, separated by denaturing gradient gel electrophoresis and sequenced. This system has proven to be very powerful in the study of mutations in cultured peripheral human lymphocytes (110), but in mice the assay is difficult to optimize, labor intensive, and may be biased by T cell growth inhibitors (such as NO•) (111).

The first mutant detection system based on the use of a transgenic system was developed by Gossen *et al.* (112)[1]. The ΦlacZ mouse contain 80 copies of the λgt10lacZ shuttle vector integrated on chromosome 3 in a head-to-tail fashion. Each individual prophage DNA has an approximate size of 47 kb including the 3126 bp lacZ gene. The mutation assay is performed by incubating purified transgenic mouse DNA with a commercially available packaging extract. Enzymes present in the extract cleave the cos sites at both ends of each prophage and package the DNA into viable λ phage particles. The individual phage can be scored for the ability to code for active ß-galactosidase by plating the phage on the indicator strain *E. coli C Δ{lac recA galE}*. The mutant selection system is based on the ability of phage transformed bacteria to grow on plates containing 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) and the inability of non-mutated phage to grow on plates containing phenyl-ß-D-galactopyranoside (P-gal) (113, 114). This system is also used for the pUR288 system (see below).

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[1]commercially sold by Hazelton Laboratories under the name MutaMouse™

The ΦlacI[2] is analogous to the ΦlacZ system. The mice harbor 80 copies of the 1080 bp lacI target gene on a lambda shuttle vector. The phage is excised from mouse DNA with the use of the same packaging extracts as ΦlacZ. Screening of the phage is carried out on *E. coli* strains harboring the lacZ gene. The assay relies on the ability of the lacI protein to repress expression of ß-galactosidase. Consequently, mutant phage produce a blue color in the presence of X-gal, which facilitates their detection in a background of white plaques (115, 116). This assay is substantially more labor-intensive than the ΦlacZ assay since a system which allows positive/negative selection in this model is currently unavailable.

The phage systems have two important limitations. First, phage systems do not detect deletions. Large deletions are likely to prevent the phage from forming plaques since the mutational target is less than 10% of the λ shuttle vector and most phage sequences are required for the phage replication and packaging. Second, phage have a relatively low efficiency of packaging which limits the number of phage that can be isolated from a given tissue. These two problems of the phage-based transgenic systems have been circumvented in the pUR288 plasmid based transgenic mouse system.

The pUR288 transgenic mouse

pUR288 transgenic mice harbor 20 copies of HindIII linearized pUR288 plasmid genomically integrated in a head-to-tail fashion. The plasmid contains the *E. coli* pBR322 origin of replication and encodes for the lacZ and ampicillin resistance genes (figure 1c-1). The plasmid can be isolated with very high efficiency by incubating HindIII-digested mouse DNA with lacI-coated magnetic beads, discarding the unbound portion and subsequently adding IPTG to separate the plasmid from the beads. The isolated plasmid is circularized by a ligation reaction and electroporated into an indicator strain (figure 1c-1). This is a highly efficient process and 2x105 colony forming units can be isolated from one microgram of DNA (117). The indicator strain used is *E. coli C (Δlac recA galE)*. This strain does not contain the lac operon (*Δlac*); is deficient in its ability to destroy differentially methylated mammalian DNA (*recA*); and contains an inactivated copy of the last gene of the galactose operon, uridine-diphospho-galactose-4-epimerase (*galE*). This last modification allows for a selection against wild type lacZ. In the presence of

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[2] commercially sold by Stratagene under the name Big Blue™

ß-galactosidase, phenyl-ß-D-galactopyranoside (P-gal) is converted to galactose. The first two genes of the galactose operon convert galactose to UDP-galactose. Inactivation of galE prevents the conversion of UDP-galactose to UDP-glucose, leading to accumulation of the toxic UDP-galactose and the death of the host cell (113, 118) (figure 1c-2). The assay is thus performed by plating one thousandth of the electroporated bacteria on ampicillin and X-gal to determine the plating efficiency and plating the rest of the bacteria on ampicillin and P-gal to identify mutations (for more information see (113, 117, 119)).

The ability of the plasmid to replicate in bacteria is dependent on the presence of the ampicillin resistance gene and the origin of replication. A deletion which removes a substantial part of the lacZ gene, or removes parts of adjacent plasmids, could still be detected in this model (figure 1c-1). Analysis of spontaneous mutants (117, 120) has demonstrated that approximately 50% of pUR288 mutants contain a deletion large enough to be detected on an agarose gel as compared to approximately 10% deletions in both ΦlacZ and ΦlacI systems (121). The relatively simple procedure, the efficient recovery and the ability of the pUR288 system to detect deletions made this system applicable for our work.

Induced mutations found *in vivo*

Of the five models discussed in the previous sections, the two phage-based systems have been used most extensively to date. From the data accumulated in these models, it is clear that interlaboratory variability is substantial and results of different studies may not be directly comparable (121, 122). Nonetheless, the systems do provide a preliminary indication of the mutagenicity of chemicals *in vivo*.

Spontaneous mutations in the hprt locus of splenic T cells are by far the least frequent ($2x10^{-6}$ in 3 month old mice (111)). The background mutant frequency in intestinal dlb-1 ($1x10^{-4}$ in young adults (123)) is much closer to the mutant frequencies found in transgenes, which typically are in the order of $1x10^{-5}$ and $1x10^{-4}$.

Spontaneous mutant frequency in transgenic mice:

System	MF x10-5				Reference
ΦlacI	liver: 2.7 - 10	spleen: 1.3 - 12.8	kidney: 1.5 - 3.7		(121)
ΦlacZ	liver: 1.0 - 7	spleen: 1.3	skin: 2.8- 3.7		(121)
pUR288	liver: 6.8	spleen: 5.4	kidney: 5.9		(117)

Responses to mutagens in the different models seem to be proportional to the background mutant frequency. In one study, the mutagenicity of N-ethyl-N-nitrosourea (ENU) was compared in the hprt locus and ΦlacI transgene. The hprt locus was somewhat more sensitive to low doses, but the slopes of the dose-response curves were found to be very similar (111). All models were validated with dose-response experiments using several potent chemical mutagens, most notably ENU.

ENU-induced mutant frequency in marker genes

gene	tissue	ENU dose (mg/kg)	MF x10-5 (control)	MF x10-5 (treated)	Reference
HPRT	T cells	40	0.18	11.6	(111)
ΦlacI			2.0	15.5	
dlb-1	small intestine	125	10.0	80.0	(123)
ΦlacZ	liver	100	1.9	3.3	(124)
		250		24.2	
ΦlacI	spleen	125	1.7	4.0	(124)
		250		9.6	
pUR288	spleen	100	5.7	40.0	(117)
		250		85.0	

Spontaneous accumulation of mutations

The accumulation of mutations with age has been studied in several mouse models. Lee *et al.* used ΦlacI mice to determine the mutant frequencies in spleens as a function of age from birth to 24 months. They found a linear increase in the mutant frequency with average values of $3.5x10^{-5}$ in newborn pups, $8.6x10^{-5}$ at three months, $10.6x10^{-5}$ at 11 months and $15.3x10^{-5}$ in 24 month old animals (125). An analogous study was performed by Ono *et al.* on ΦlacZ mice. They found an average mutant frequency of $3.2x10^{-5}$ in newborn pups, $5.2x10^{-5}$ at two months and $8.3x10^{-5}$ at one year (126). Heddle's group published two conflicting reports on the accumulation of mutations in the dlb-1 locus as a function of age. In one study they reported a five-fold increase in mutant frequency between the ages of 6 and 40 months (from $10x10^{-5}$ to $70x10^{-5}$, (123)) and in the other study a two-fold increase (from $20x10^{-5}$ to $40x10^{-5}$, (121)). Early results from pUR288 transgenic mice support the observations of the two phage models. In pUR288 liver and spleen mutant frequencies rise from $6x10^{-5}$ and $7x10^{-5}$ in six week old mice to $20x10^{-5}$ and $12x10^{-5}$, respectively, in 18 month old mice (120). All three transgenic models converge on a two fold per year increase in spontaneous mutant frequency. It should be noted that higher mutant frequencies have been reported for pUR288 as compared to equivalent ΦlacZ and ΦlacI. This is likely to be related to the ability of the pUR288 model to better detect deletions.

The spontaneous mutant spectra in ΦlacI and ΦlacZ have been assembled by Zhang *et al.* (121). They report that approximately 80% of spontaneous mutants were base substitutions (single or multiple), 10% were small deletions, and 10% were frameshift mutations. Base pair substitutions in ΦlacZ liver occurred most often in CpG islands, with GC→AT being most common (12/27,45%) (127). The mutant spectrum in ΦlacI spleen has been studied by two different groups. Both groups found that base pair substitutions accounted for 80% of the mutants. In one study the most common base pair substitution was GC→TA (6/22, 27%) (121) and in the other GC→AT (7/16, 45%) (115). In the pUR288 transgenic mouse large deletions accounted for 13/29 (45%) of the mutations in the spleen (120). Only a few base substitution mutants were sequenced, of which 3/14

(21%) were GC→AT (M.E.T.I. Boerringter, personal communication). Overall, few studies to date have attempted to sequence mutants induced *in vivo*, and a definitive spontaneous mutant spectrum cannot be constructed based on available data.

Experiments over the last five years have validated the use of transgenic mice as indicators of mutagenicity of both chemical and spontaneous origin (aging). While experiments can only be internally compared, the spontaneous mutant frequencies were generally found to be between $1x10^{-5}$ and $10x10^{-5}$ with a value of $5.4x10^{-5}$ for the pUR288 spleen. The pUR288 system, while less frequently used, was chosen for this study because it is capable of detecting deletions and only small amounts of DNA (50 μg) are required for the assay.

Isolation of pUR288 plasmid from host DNA

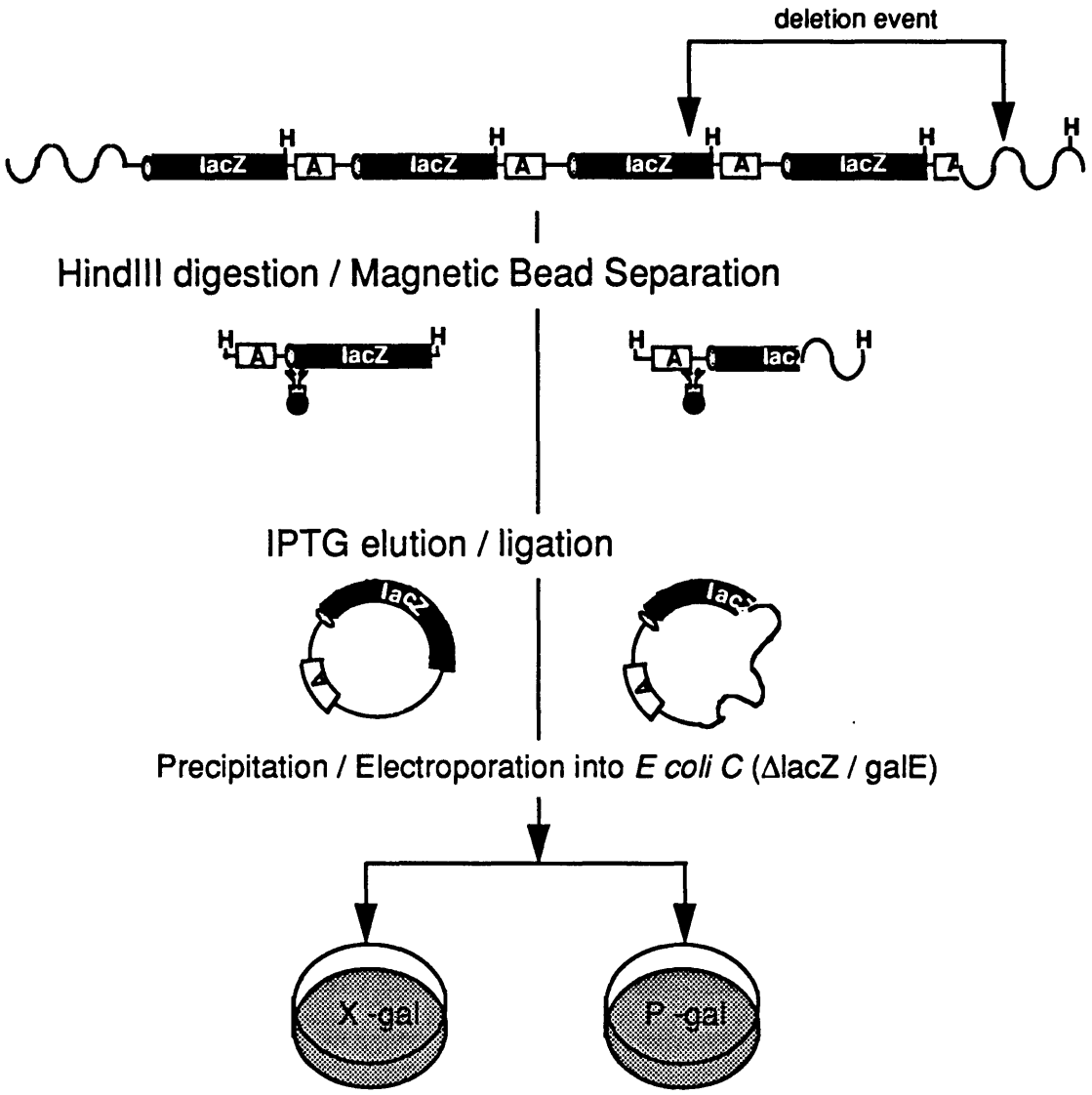

Fig 1c-1: Schematic depiction of plasmid rescue from pUR288 transgenic mouse DNA. HindIII digestion releases single copies of the plasmid from the tandem array flanked by genomic mouse sequences. Plasmid sequences are then purified by magnetic beads coupled to lacI. Circular forms of the plasmid are obtained after elution and ligation. The plasmid is electroporated into the indicator bacterial strain and plated on selective and non selective media. (Figure adapted from Dolle, *et. al.* Mutagenesis 11:111-118)

Basis for positive selection of lacZ plasmids

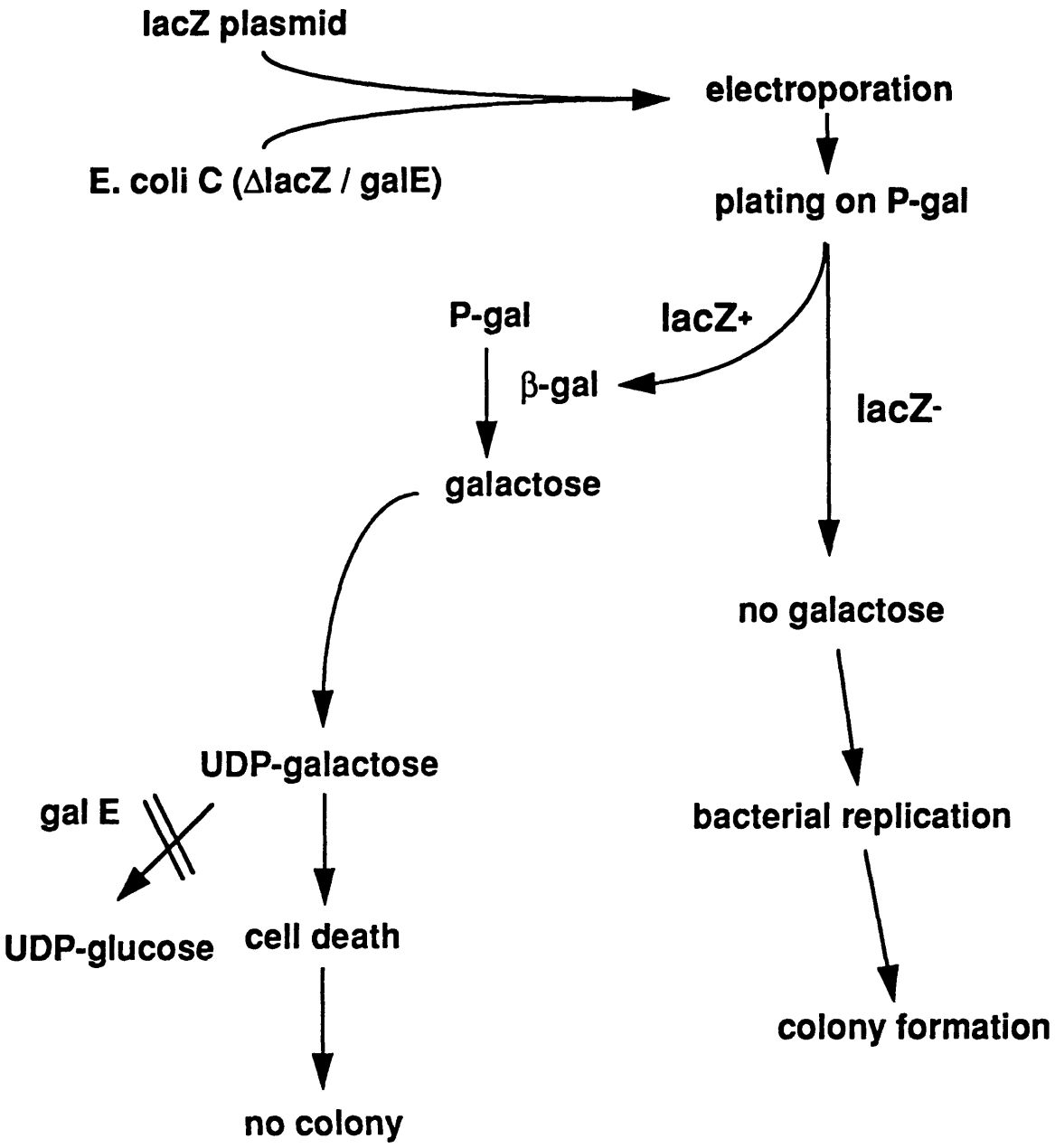

Fig 1c-2: Positive selection system for lacZ⁻ plasmids. *E. coli C ΔlacZ / galE* cells expressing the wildtype lacZ gene are unable to grow as they cannot convert the toxic UDP-galactose into harmless UDP-glucose. Only bacteria containing a mutant lacZ plasmid will be able to form colonies. (Figure adapted from Martus, *et al.* Mutation Res. 338:203-213)

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2. Nitric oxide production in SJL mice bearing the RcsX lymphoma: A model for *in vivo* toxicological evaluation of NO·

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Abstract

SJL mice spontaneously develop Pre-B cell lymphoma that we hypothesized might stimulate macrophages to produce nitric oxide ($NO^{\bullet}$). Transplantation of an aggressive lymphoma (RcsX) was used to induce tumor formation. Urinary nitrate excretion was measured as an index of $NO^{\bullet}$ production and was found to increase 50 fold by 13 days after tumor injection. $NO^{\bullet}$ production was prevented by the addition of a nitric oxide synthase (NOS) inhibitor. The expression of inducible nitric oxide synthase (iNOS) in various tissues was estimated by western blotting and localized by immunohistochemistry. The synthase was detected in the spleen, lymph nodes and liver of treated but not control mice. To assess whether the iNOS-staining cells were macrophages, spleen sections from RcsX bearing animals were co-stained with anti-iNOS antibody and the anti-macrophage antibody moma-2. Expression of iNOS was found to be limited to a subset of the macrophage population. The concentration of gamma interferon, a cytokine known to induce $NO^{\bullet}$ production by macrophages, in the serum of tumor-bearing mice, was measured and found to be elevated 25-fold above untreated mice.

The ability of RcsX-activated macrophages to inhibit splenocyte growth in primary culture was estimated and macrophage-derived $NO^{\bullet}$ was found to inhibit cell division 10 fold. Our findings demonstrate that RcsX cells stimulate $NO^{\bullet}$ production by macrophages in the spleen and lymph nodes of SJL mice, and we believe this experimental model will prove useful for study of the toxicological effects of $NO^{\bullet}$ under physiological conditions.

Introduction

The free radical nitric oxide ($NO^{\bullet}$) has multiple functions in mammalian physiology. At physiological levels, it is associated with regulatory functions, such as long-term potentiation in the brain (1) and vasodilation (2). At higher levels, it has been associated with tissue damage (3), tumoricidal (4) and bactericidal (5) effects. It also has been shown to be cytostatic to T cells and tumor cells *in vitro* (4) and has been reported to induce apoptosis (6). $NO^{\bullet}$ mediates the oxidant injury caused by paraquat in isolated guinea pig lungs (7), and possesses tumoricidal activity against transplanted tumors in mice (8). Mice lacking the inducible isozyme of nitric oxide synthase (iNOS) are deficient in the ability to resist bacterial infection (9).

Long-term endogenous production of $NO^{\bullet}$ has been suspected of causing adverse effects *in vivo*. 3-Nitrotyrosine, a biomarker of such production, has been detected in tissue proteins of patients with diseases thought to have autoimmune components, such as atherosclerosis (10) and rheumatoid arthritis (11). Evidence for involvement of $NO^{\bullet}$ in autoimmune responses has also been produced in experimental animals, including MLR-lpr/lpr mice, in which development of spontaneous immune complex glomerulonephritis was prevented by administration of NOS inhibitors (12). In SJL mice, it was shown to play a role in the pathology of experimental autoimmune encephalomyelitis (13), and have been used as an experimental model for the disease (14).

SJL mice were derived from the Swiss Webster strain (15), and exhibit multiple immunological disorders, paraproteinemia (16) and myositis (27). They are suspected of having defective suppressor T cell function (18), and during the first year of life, spontaneously develop lymphomas arising in germinal lymphoid centers (15, 19). The lymphomas are of B cell lineage (20, 21) and present an MMTV-LTR derived superantigen on their surface. This superantigen stimulates $Vb16^{+}$ T cells (22) which in response secrete cytokines required for growth of the lymphoma cells *in vivo* and *in vitro* (23, 24). The tumor may also require host macrophages for *in vivo* growth (25) and primary explant co-culture of the tumor with SJL splenocytes is accompanied by production of gamma interferon (26).

We previously demonstrated (27) overproduction of $NO^{\bullet}$ in spontaneous myositis concurrently involving macrophage infiltration into skeletal muscle tissue in SJL mice. These findings, together with the spontaneous development of autoimmune disease,

stimulation of the host immune system by transplantation of the lymphoma and the production of gamma interferon by tumor cells in primary culture suggested the possibility that transplantation of the lymphoma into SJL mice might induce NO· production by host macrophages. The RcsX tumor cell line (23) was derived by successive transplantation of an SJL lymphoma into syngeneic host animals (21, 24). Intraperitoneal injection of RcsX cells into SJL mice leads to rapid growth of tumor as well as host T cells in the lymph nodes, spleen and liver, resulting in morbidity 15 days later. We report here results supporting the conclusion that large amounts of nitric oxide are indeed produced by macrophages in the spleen and lymph nodes of SJL mice bearing the RcsX tumor. This experimental model will be useful for further characterization of the toxicology of NO· *in vivo*.

Materials and Methods

Animal Experiments. Female SJL mice, fourteen weeks old (Jackson Labs, Bar Harbor, ME), were fed a low nitrate control diet (AYN-76A, Bioserve, Frenchtown, NJ) for four weeks to minimize the background rate of nitrate excretion. The mice were then weighed, placed individually in metabolic cages and their drinking water was replaced with 30 mM solution of the nitric oxide inhibitor N^G-methyl-L-arginine acetate (NMA) (Chem Biochem Research, Salt Lake City, UT) or 30 mM ammonium acetate (Sigma Chemical Co., St Louis, MO). Two days later, six mice receiving NMA and six mice receiving ammonium acetate were each injected intraperitoneally with 0.2 ml PBS containing 10^7 cells of the RcsX line (kindly provided by N. Ponzio, University of New Jersey Medical Center, Newark NJ), isolated from lymph nodes of mice bearing the actively growing tumor. Urine was collected on alternate days into tubes containing 0.5 ml 0.5 M NaOH to inhibit bacterial growth. Total urinary nitrate concentration was determined as previously described (28) and normalized to body weight. All animals were weighed and killed by CO_2 asphyxiation 14 days after injection of cells. Spleen, liver, peripheral lymph nodes, kidneys, thigh skeletal muscle, heart and serum were removed, and portions of each tissue were fixed in formalin and embedded in paraffin; the remainder was frozen in liquid nitrogen for further analysis.

Western blotting. Inducible nitric oxide synthase (iNOS) was isolated from tissues according to the protocol described by Oguchi *et al.* (29) with minor modifications. Tissues were homogenized in buffer A {50 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, (all from Sigma)} and centrifuged at 100,000 x g for 60 min at 4°C. Protein of the cytosolic fraction (500 mg) were incubated with 2', 5' ADP agarose (50:1) for 30 minutes at 4°C. The agarose was washed three times with buffer A and iNOS was eluted with buffer A containing 10 mM NADPH. The eluted protein was concentrated on a Rotovap (Savant, Farmingdale, NY) and loaded onto an 8% SDS-PAGE gel, and transferred to a nitrocellulose filter (Schleicher & Schuell, Keene NH) overnight. The membrane was blocked for 2 hrs with 3% bovine serum albumin (Biowhittaker, Walkersville, NY) in PBS at room temperature and then incubated with rabbit anti-mouse nitric oxide synthase (5 μg/ml, a gift from Dr. M. Marletta, Univ. of Michigan, Ann Arbor, MI) in blocking buffer for 2 hrs. The membrane was washed then incubated with 1 μg/ml mouse anti-rabbit alkaline phosphatase and developed using a substrate kit (Bio Rad, Hercules, CA) using

5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitro-blue tetrazolium chloride (NBT) according to manufacturer's instructions.

Immunohistochemistry. Immunohistochemical staining for iNOS was performed on sections prepared from formalin-fixed, paraffin-embedded tissues. Sections were deparaffinized in xylene, then rehydrated by passage through decreasing concentrations of ethanol in water. Sections were incubated successively with avidin and biotin (Dako Corp, Carpinteria CA) for 10 min to block reagent binding to endogenous biotin and with CAS Block (Zymed, South San Francisco, CA) for 15 min to block nonspecific binding. Sections were incubated with polyclonal rabbit anti-mouse iNOS antiserum (10 mg/ml) in PBS for 2 hrs at room temperature. Thereafter goat anti-rabbit IgG conjugated to biotin (Dako) was incubated with the tissues for 10 min., followed by incubation with alkaline phosphatase-conjugated strepavidin (Dako) for 10 min. Color was developed using fast red as the chromogen, resulting in a bright red precipitate at antigen sites. Sections were counterstained with Mayer's hematoxylin.

Double staining with the macrophage-specific antibody moma-2 and anti-iNOS was performed on frozen sections. Tissue sections were fixed in cold methanol (4°C) for 15 minutes, and incubated with rat anti-mouse moma-2 antibody (1:2.5; Serotec, UK) for 1 hr and then in goat $F(ab)_2$ anti-rat IgG-FITC (Caltag, South San Francisco, CA) for 10 minutes. The slides were dipped in formalin, rinsed for five minutes in running water and incubated with iNOS and goat anti-rabbit IgG as described above, then with 2 μg/ml strepavidin Cy-3 (Caltag) for 10 minutes.

Gamma-interferon assay. Blood (1 ml) was collected by heart puncture from 6 tumor-bearing and 6 control mice at the time they were killed, allowed to coagulate and serum was separated by centrifugation. Serum levels of gamma interferon were measured by ELISA (R&D systems, Minneapolis MN) according to manufacturer instructions.

Cell culture experiment. Lymph nodes and spleen were aseptically isolated from mice 14 days after the injection of RcsX cells. A single cell suspension was prepared by mincing and repeated pipetting in cold phosphate buffered saline. Cells were pelleted and washed three times and stained with trypan blue to assess viability. Only preparations containing more than 95% dye-excluding cells were used. Cells were cultured in 96 well plates (Falcon, Beckton Dickinson, Oxnard CA), at a density of 40,000 cells/well in 0.2 ml medium containing Isocaves modified minimal essential medium (2 mM L-glutamine, 0.2 U/ml penicillin, 0.2 U/ml streptomycin, 1 mM sodium pyruvate, 1 mM oxaloacetic acid, 15% fetal calf serum, Biowhittaker, Walkersville, NY; and 0.2 U/ml insulin and 50 μM

2-mercaptoethanol, Sigma). After four hrs, nonadherent cells were transferred to another well and NMA was added to a final concentration of 1 mM. ^{3}H-thymidine (DuPont NEN, Cambridge, MA) was added at a level of one microcurie per well and allowed to remain in the medium for a period of 24 hrs. Cells were then harvested with an automated cell harvester (Sketron, Sterling VA) and incorporated radioactivity was measured by scintillation counting (LBK, Gaithersburg MD).

Results

RcsX-bearing SJL mice excrete elevated amounts of urinary nitrate; excretion is inhibited by the iNOS inhibitor NMA. To test the hypothesis that NO• would be overproduced in tumor-bearing SJL mice, animals were each injected with RcsX tumor cells as described in Materials and Methods, and total NO• production was monitored by measuring urinary excretion of nitrate, its ultimate metabolic product in mammals (30). Treated mice showed a time-dependent increase in urinary nitrate excretion ($P < 0.05$ by t-test on day 6, Fig. 1), beginning four days after cells were injected, culminating in a 50-fold increase on the fourteenth day of the experiment. Treated mice also receiving NMA showed no elevation in nitrate excretion until the last two days of the experiment. Only minor variations in nitrate excretion occurred in untreated and NMA-treated control animals throughout the experiment. These results demonstrated that growth of RcsX cells was temporally associated with significant elevation of nitric oxide production in SJL mice.

Administration of NMA alone did not affect body weights of the animals, and no differences were observed in the weights or gross appearance of organs from mice receiving NMA and those from mice receiving ammonium acetate in the drinking water. Spleens from RcsX-injected mice receiving ammonium acetate weighed 1.43 ± 0.34 grams and those from treated mice receiving NMA weighed 1.33 ± 0.31 grams; peripheral lymph nodes from animals receiving the same treatments weighed 0.77 ± 0.25 and 0.81 ± 0.21, respectively.

Inducible nitric oxide synthase is expressed in lymphoid tissue and liver of tumor-bearing mice. In order to localize the nitric oxide synthase isozyme serving as the ultimate source of urinary nitrate observed in the above animals, presence of the endothelial, brain and inducible isoforms of the enzyme was determined by western blotting of liver, spleen, kidney, lymph node, heart, brain and muscle. Detectable levels of iNOS were present in spleen, lymph nodes and liver of RcsX-bearing animals, but not in control mice (Fig. 2). Neither brain NOS nor endothelial NOS was detectable in any tissues of the tumor-bearing or control animals. These results support the conclusion that nitric oxide was produced by the inducible isoform of nitric oxide synthase in tumor-bearing animals.

Inducible nitric oxide synthase protein is localized to small subpopulations of tumor cells in lymph nodes and spleen, as well as in metastatic cells in

liver. Immunohistochemical staining for iNOS was performed in order to determine the cellular localization of nitric oxide production, as described in Materials and Methods. The iNOS protein was detected in cells scattered throughout the lymph nodes and spleen in RcsX-treated (Fig. 3A) but not control (Fig. 3B) mice. Staining was distinctly localized in individual cells estimated to represent collectively less then 5% of the total cell population. In the spleen, staining was concentrated in, but not limited to, follicular regions (Fig. 3A). Cells staining for iNOS typically were larger than surrounding cells and contained a relatively large amount of cytoplasm and vacuoles (Fig. 3C, 3D), an appearance consistent with the morphology of macrophages. In the liver, staining was limited to islands of metastatic cells located around central veins (Fig. 3E). Some tumor cells infiltrating into the kidney were also stained (Fig. 3F), but in both liver and kidney very few cells contained detectable levels of iNOS, and those that were present were morphologically similar to iNOS-positive cells in the spleen and lymph nodes. Liver parenchyma was devoid of iNOS-containing cells and the kidney parenchyma stained only weakly. These results indicated that iNOS-expressing cells were localized primarily within lymphoid tissues of RcsX tumor-bearing mice, and further suggested that a small subpopulation of cells present in the tumor mass was responsible for the observed elevation of nitric oxide production.

iNOS expression co-localizes with the macrophage specific moma-2 antigen. To determine whether cells expressing iNOS were macrophages, spleen sections isolated from tumor-bearing mice were stained with the rat anti-mouse macrophage antibody moma-2 (31) as well as with anti-iNOS as described in Materials and Methods. Expression of iNOS was limited to a subpopulation of moma-2 positive cells (Fig. 4), consistent with the conclusion that macrophages were indeed the major source of nitric oxide in these animals.

Serum levels of gamma-interferon are elevated in tumor-bearing mice. Because gamma-interferon has been implicated in the induction of iNOS expression in macrophages *in vitro* and *in vivo*, levels of this cytokine in serum from RcsX bearing SJL mice were measured, and found to be elevated by 25-fold compared to those in controls (Fig. 5). NMA treatment had no detectable effect in either tumor-bearing or control mice. This response provides further supportive evidence concerning a possible mechanism of iNOS induction in macrophages of RcsX tumor-bearing mice.

Adherent cells isolated from RcsX tumor produce nitric oxide in primary culture. Nitric oxide has been shown to inhibit growth of spleen cells *in vitro* (32, 33),

and we utilized this property to provide additional evidence concerning the source of nitric oxide in this experimental system. Primary cultures of splenocytes from RcsX-treated mice showed no detectable ^{3}H-thymidine incorporation over a 48 hr period (Fig. 6). However, addition of NMA to the culture medium resulted in a substantial increase in cell division (Fig. 6). Suspensions of spleen cells were cultured in microwell plates for four hours, during which time macrophages were expected to become adherent. Nonadherent cells (estimated to represent about 80-90% of the total) were then transferred into a well devoid of adherent cells. Separation of cells in this way (i.e., culture of splenocytes in the absence of macrophages) led to increased thymidine incorporation by nonadherent cells, which was further enhanced by incorporation of NMA in the medium (Fig. 6). These results indicate that adherent cells (primarily macrophages) were responsible for inhibiting the growth of nonadherent cells, putatively through nitric oxide production, thus providing additional evidence that macrophages were the source of $NO^{\bullet}$ in this experimental model.

Fig 2.1 Nitrate excretion by RcsX bearing SJL mice and controls. Twelve SJL mice were injected i.p. on day 0 with 10^7 RcsX cells. Six RcsX-bearing and six control mice received 30 mM NMA in drinking water starting on day 2. All tumor-bearing mice were killed on day 13.

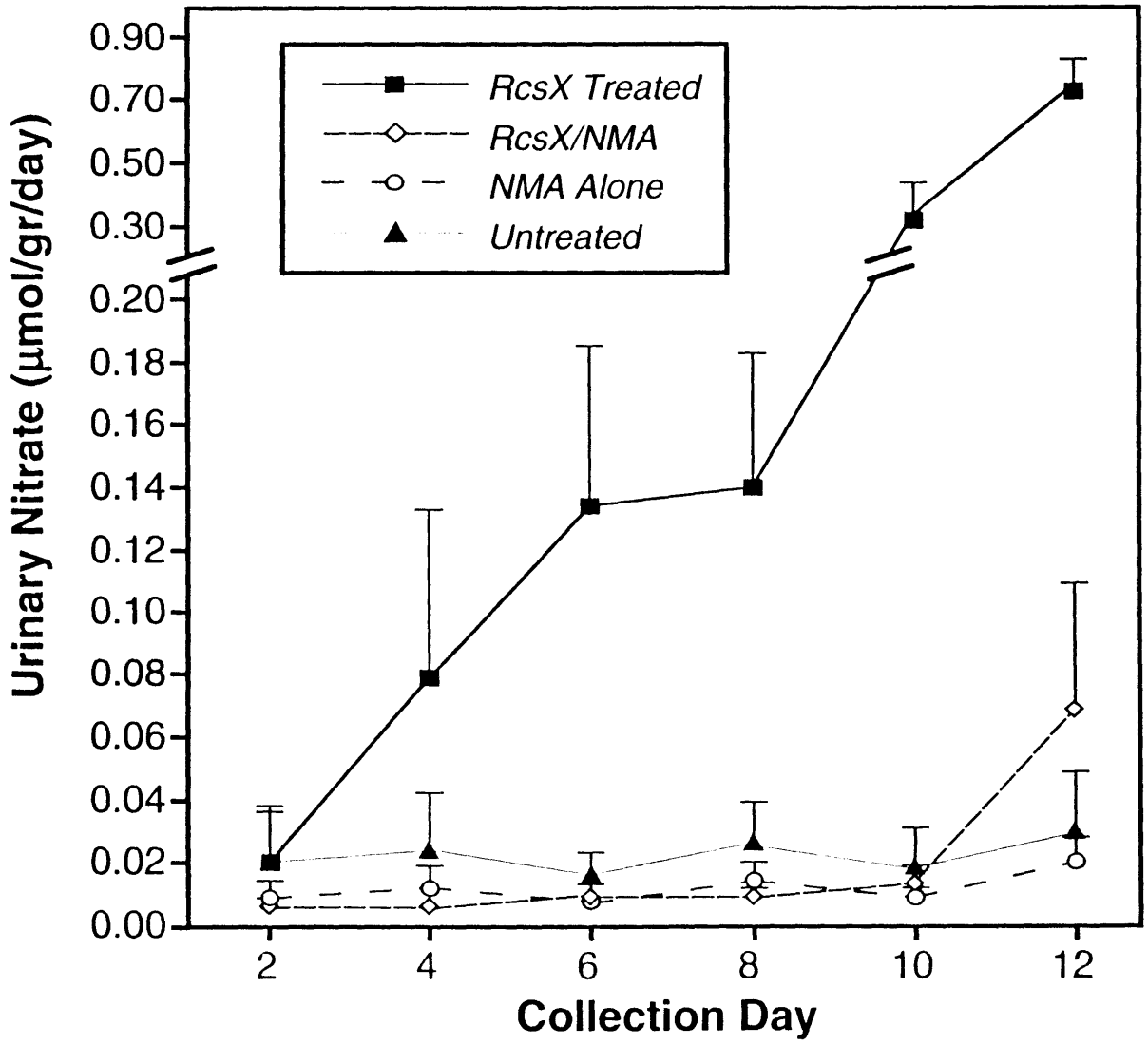
Fig 2.2 Western blot analysis of iNOS expression. Tissues from an RcsX tumor bearing SJL mouse were isolated 14 days after injection of 10^7 RcsX cells and western blotting was performed as described in Materials and Methods. Activated macrophages were used as positive control. Control mice had no detectable iNOS expression in any of the tissues analyzed.

Fig 2.3 Immunohistochemical analysis of iNOS expression in tissues of RcsX tumor-bearing SJL mice. Sections were prepared and stained as described in Materials and Methods. A) Spleen of RcsX-bearing mouse (100x); B) Spleen of control mouse (100x); C) Spleen of RcsX-bearing mouse (400x); D) Spleen of RcsX-bearing mouse (1000x); E) Liver of RcsX-bearing mouse (400x); F) Kidney of RcsX tumor-bearing mouse (400x).

Fig 2.4 iNOS and moma-2 expression in spleen of RcsX tumor-bearing SJL Mice. The section was stained with moma-2 (FITC-green) and iNOS (Cy3-red) as described in Materials and Methods (1000x).

Fig 2.5 Serum levels of gamma-interferon in tumor-bearing SJL mice.

Fig 2.6 Growth of Splenocytes isolated from RcsX tumor-bearing SJL mice in primary culture. Cells, 40,000 per well, were cultured in quadruplicate in a 96 well plate as described in Materials and Methods. After 4 hours non-adherent cells were transferred to another well and 1 mM NMA was added. ^{3}H-Thymidine incorporation was added 24 hours before harvest in order to quantify growth.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Urinary Nitrate (µmol/gr/day)
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2
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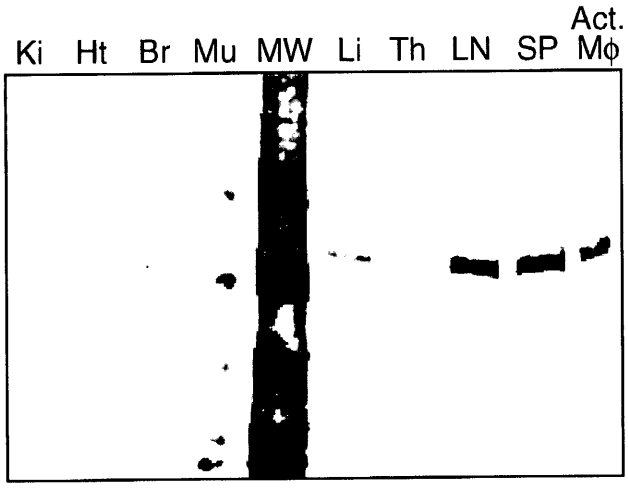
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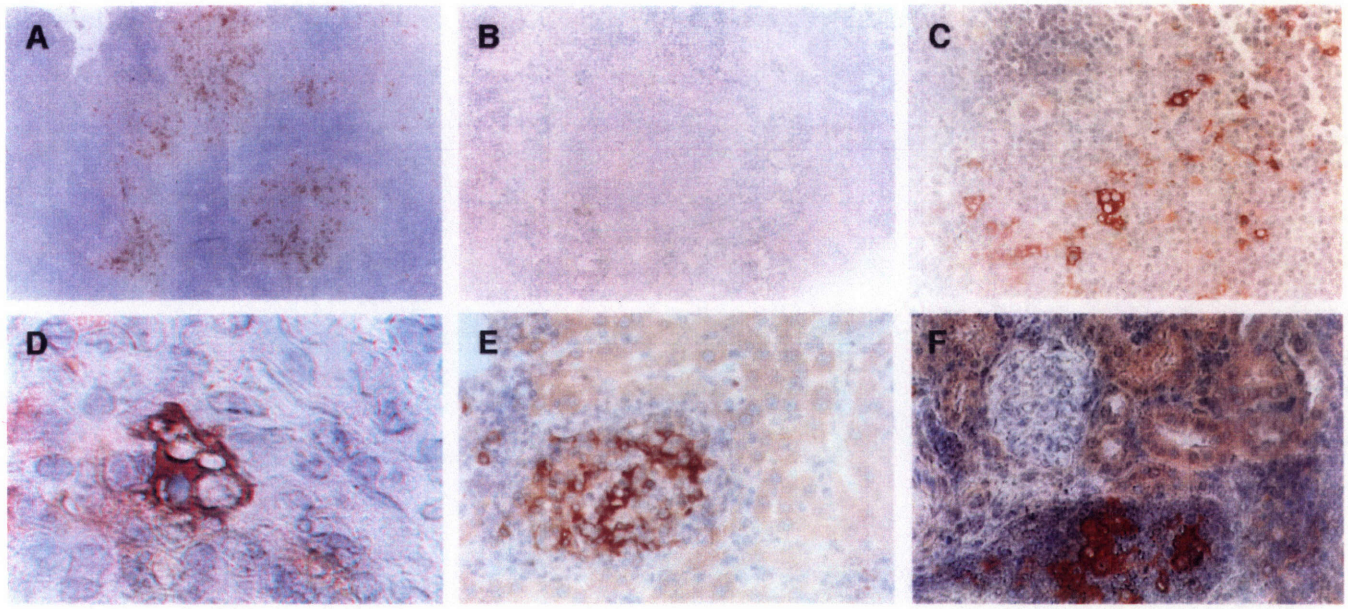
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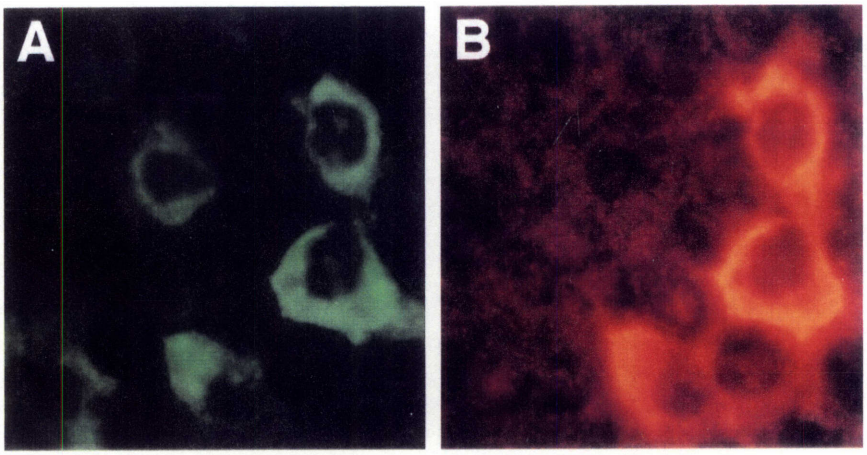
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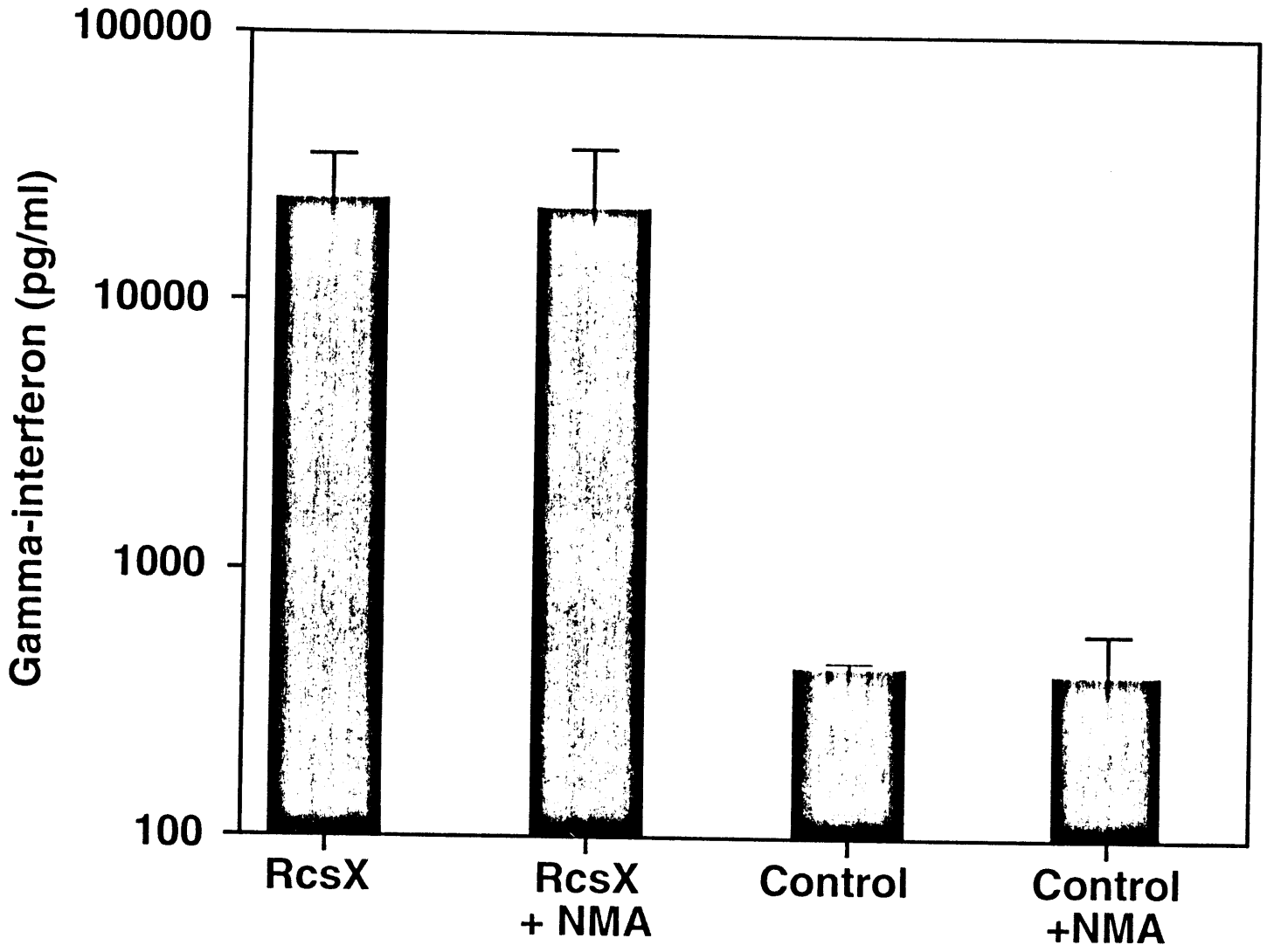
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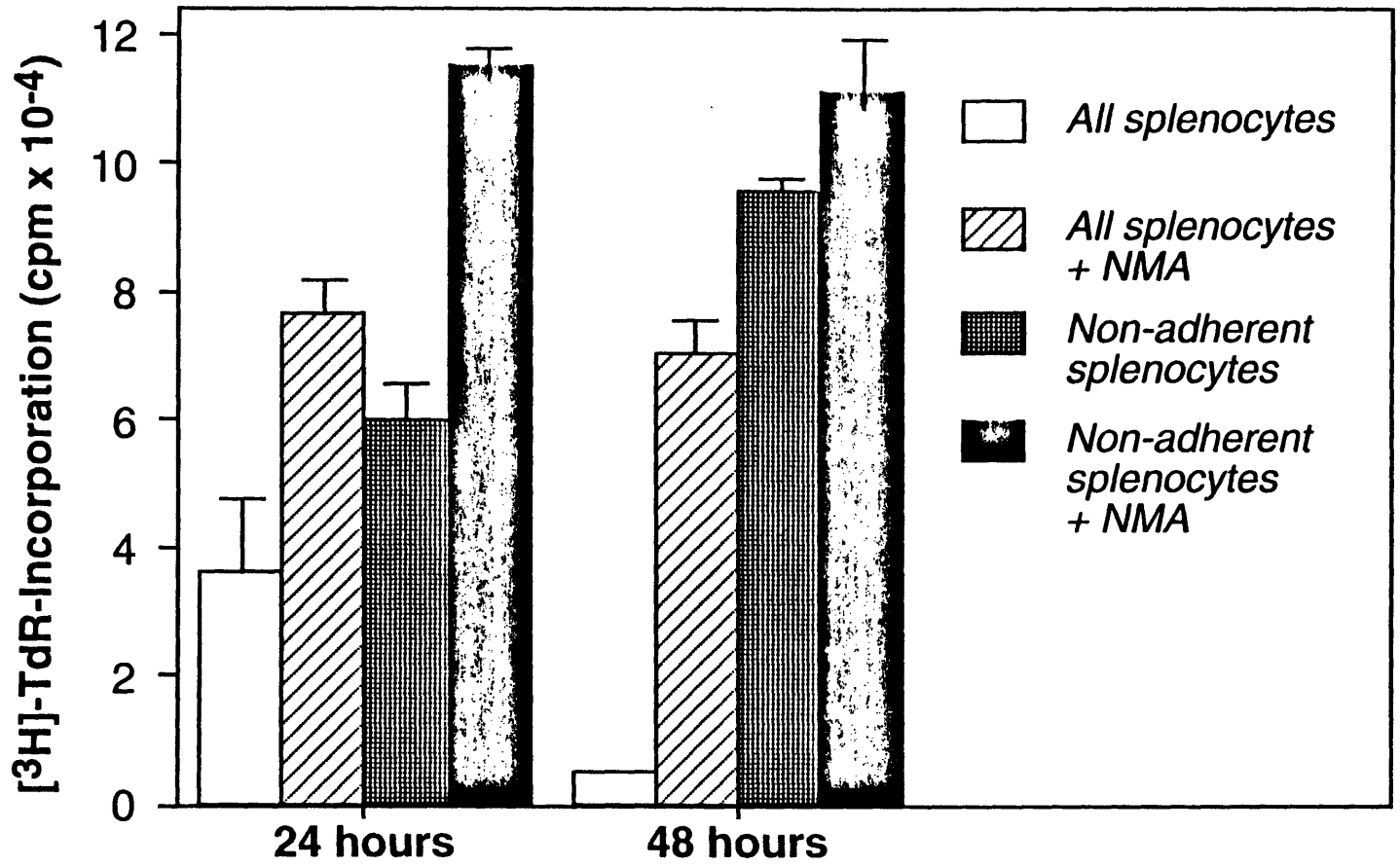
+NMA


[3H]-TdR-Incorporation (cpm x 10-4)
12
10
8
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4
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24 hours
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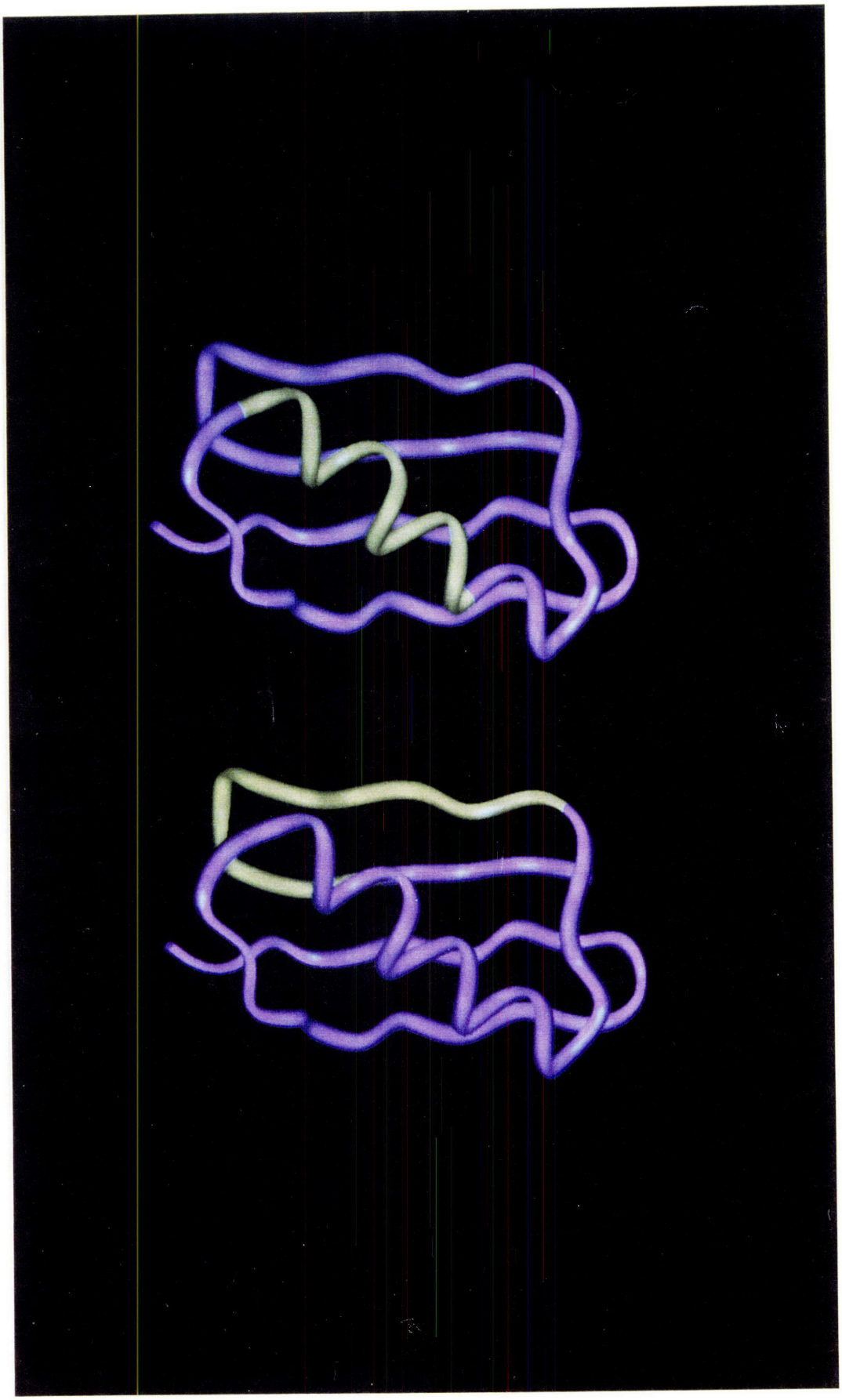
All splenocytes + NMA
Non-adherent splenocytes
Non-adherent splenocytes + NMA

Discussion

The objective of this work was to characterize an animal model in which NO• production can be physiologically induced over time periods and at levels sufficient for investigation of damage to cellular macromolecules. Excretion of nitrate, the final metabolite of nitric oxide *in vivo*, was measured in urine of RcsX tumor-bearing animals, and results (Fig. 1) demonstrated a 50-fold elevation in endogenous production of the radical. In the context of sites of potential NO•-induced damage, cells of the spleen and lymph nodes would be exposed to higher doses than those of other tissues, inasmuch as production was localized in those tissues. Further, localized production in a small subpopulation of cells would suggest that gradients of nitric oxide concentration likely existed within those tissues, in which exposure of individual target cells would depend on their position in relation to generating cells.

Although administration of NMA in drinking water effectively prevented the production of NO• through most of the experiment, relatively small amounts were produced during the final two days, possibly due to induction of iNOS. We believe the time-dependent increase in amount of NO• produced was related to increased mass of the spleen and lymph nodes, not to differential expression of iNOS during the experiment, on the basis of invariate intensity of staining in western blots and immunohistochemical analyses of tissues isolated at different stages of the experiment (data not shown). It should be noted that nitrate excretion in control mice was also decreased by NMA. While this may have been due to inhibition of background levels of endothelial or brain NOS, it was more likely associated with the spontaneous development of myositis as we previously reported (27).

Induction of iNOS expression in lymphoid tissue was limited to a small subpopulation of splenocytes and lymph node cells (Fig. 3). In the spleen, iNOS-expressing cells were concentrated in follicular regions, the zones implicated in the presentation of antigens (34, 35). This localization could potentially be related to the action of the superantigen, which mimics antigen presentation to T cells, or to differential regulation of macrophages in follicular and non-follicular regions. Differential regulation of macrophages in different compartments is reflected in the antigen profile presented by macrophages. While the moma-2 antibody recognized iNOS-expressing cells (Fig. 4), two other anti-macrophage antibodies, mac-1 and F4/80, did not (data not shown). In the liver, iNOS expression was observed in macrophages comprising metastatic islands, but not in



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3. Nitrotyrosine Formation, Apoptosis and Oxidative Damage: Relationships to Nitric Oxide Production in SJL Mice Bearing the RcsX Tumor

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Abstract

In SJL mice, growth of RcsX lymphoma cells results in activation of macrophages in the spleen and lymph nodes to produce high levels of nitric oxide (NO•). We used this experimental model system to study the toxicology of nitric oxide *in vivo*. To characterize spatial relationships between sites of nitric oxide production and tissue damage, immunohistochemical techniques were developed for simultaneous detection of inducible nitric oxide synthase (iNOS), Nitrotyrosine and apoptosis in spleen and lymph nodes of tumor-bearing animals. Elevated expression of iNOS, presumed to reflect increased NO• production, was associated with a significant increase in frequency of apoptotic nuclei. Both apoptotic nuclei and nitrotyrosine-staining were found in cells juxtaposed to iNOS-expressing (i.e., NO•-producing) macrophages and also within the macrophages themselves. To assess the extent of DNA damage associated with the response, 8-oxoguanine levels were quantified in DNA extracted from spleen of tumor-bearing mice. No increase in levels of this marker of oxidative DNA damage was found in tissues in which apoptosis and nitrotyrosine levels were highly elevated within specific subsets of cells. Collectively, our results indicate that under the pathophysiological conditions existing in the RcsX tumor-bearing SJL mouse, cellular damage caused by nitric oxide and/or other reactive species produced by activated macrophages is highly localized within cells in close proximity to the activated macrophages.

Introduction

An association between chronic inflammation and risk for certain malignancies has been hypothesized to be indicative of a causal relationship (1, 2). Central to the hypothesis is the observation that under inflammatory conditions macrophages and neutrophils migrate to the site of inflammation and are activated to produce reactive radicals that in turn facilitate the carcinogenic process (3, 4). The free radical nitric oxide (NO•), which has multiple regulatory functions when secreted in low levels by the NOS1 and NOS3 isozymes by a variety of cells (for reviews see (5, 6)), has an important role in mammalian defense mechanisms when produced in large amounts by the NOS2 (inducible nitric oxide synthase; iNOS) isozyme in macrophages (7, 8). Production of NO• by macrophages, together with direct evidence for its presence in several models of inflammation-associated carcinogenesis (9-11) have suggested that it may be directly involved in inflammation-mediated carcinogenesis.

Recent studies of chemical and toxicological properties of nitric oxide support this suggestion. Nitric oxide produced by macrophages has been shown to cause DNA oxidation, deamination and strand break formation (12, 13), and is mutagenic both to plasmids and to TK6 human lymphoblastoid cells (12, 14, 15). Furthermore, it has been shown to cause cytostasis in cultured T cells (7) and apoptosis in macrophages (16, 17) and pancreatic ß-cells (18). Nitric oxide can react with superoxide anion, another macrophage-derived radical, to form peroxynitrite (19), which has been shown to produce oxidative DNA damage, nitration of proteins and nucleic acids and cell killing *in vitro* (20, 21). Evidence also supports an *in vivo* role for peroxynitrite in ischemia/reperfusion injury and autoimmune tissue damage (22, 23).

While progress has been made in defining pathways through which nitric oxide acts in cell culture and cell-free systems, it has been more difficult to construct models for the complex interactions governing the consequences of its production in intact animals. We recently reported development of an experimental model system to study the toxicology of NO• *in vivo* (24, 25), in which young SJL mice were injected with cells of the RcsX transplantable pre-B cell lymphoma. These cells express a superantigen capable of activating the immune system of host animals (26), and in the course of the immune response iNOS expression was induced in macrophages located in the spleen and lymph nodes, resulting in a 50-fold increase in NO• production 14 days after tumor cell injection. In experiments reported here, to characterize spatial relationships between sites of nitric

oxide production and tissue damage, immunohistochemical techniques were developed for simultaneous detection of inducible nitric oxide synthase (iNOS), nitrotyrosine and apoptosis in spleen and lymph nodes of tumor-bearing animals. Collectively, results obtained indicate that under the pathophysiological conditions existing in the RcsX tumor-bearing SJL mouse, cellular damage caused by nitric oxide and/or other reactive species produced by activated macrophages is highly localized within cells located in close proximity to the activated macrophages.

Materials and Methods

Animal Experiment

Female SJL mice, fourteen weeks old (Jackson Labs, Bar Harbor, ME), were fed a low nitrate control diet (AYN-76A, Bioserve, Frenchtown, NJ) for four weeks to minimize the background rate of nitrate excretion. The mice were then weighed, placed individually in metabolic cages and their drinking water was replaced with 30 mM N^G-methyl L-arginine acetate (NMA, Chem Biochem Research, Salt Lake City, UT) or 30 mM ammonium acetate (Sigma Chemical Co., St Louis, MO). Two days later, six mice receiving NMA and six mice receiving ammonium acetate were each injected intraperitoneally with 10^7 cells of the RcsX line in PBS (kindly supplied by N. Ponzio, University of New Jersey Medical Center, Newark NJ), passaged by isolation from lymph nodes from mice bearing the tumor. Urine was collected on alternate days into tubes containing 0.5 ml 0.5 M NaOH to inhibit bacterial growth. Total urinary nitrate concentration was determined as previously described (27) and normalized to body weight. All animals were weighed and killed 14 days after injection of RcsX cells. Spleen, liver, peripheral lymph nodes and kidneys were removed from all animals. A section of each tissue was fixed in formalin for no more than 6 hours and embedded in paraffin. Another section was embedded in OCT compound (Miles, Elkhart, IN), placed in liquid nitrogen to produce frozen sections slides. The remaining tissue was frozen in liquid nitrogen and used in DNA damage analysis.

Analysis of Apoptosis

Apoptotic nuclei were detected by the terminal deoxynucleotide transferase mediated dUTP nick end-labeling (TUNEL) procedure on formalin-fixed tissues with a commercial kit (Apoptag, Oncor, Gaithersburg, MD) according to manufacturer's instructions. The extent of apoptosis was estimated by counting, under 400X magnification, the number of apoptotic nuclei in 5 randomly selected fields of a representative section of one lymph node from each of five mice per treatment group (RcsX, RcsX + NMA, control).

When TUNEL and iNOS immunohistochemistry were performed on the same section, TUNEL analysis was done first, followed by the iNOS procedure as described below. The following modifications had to be introduced into the recommended TUNEL procedure in order to permit efficient iNOS detection. Proteins were not digested with proteinase K as recommended; to minimize background staining, the anti-digoxigenin antibody supplied was diluted four-fold in PBS; and development with diaminobenzamide was allowed to proceed for no more than three minutes (rather than five minutes as

recommended) to minimize background.

Nitrotyrosine and iNOS Immunohistochemistry

Combined nitrotyrosine and iNOS immunohistochemistry analysis was performed on frozen sections only. Tissue sections were fixed in formalin for one minute, then washed for five minutes in running water. They were then incubated successively with Peroxoblock (Zymed, South San Francisco, CA) for 45 seconds to block peroxidase activity, avidin and biotin (Dako Corp, Carpinteria CA) for 10 min to block reagent binding to endogenous biotin, and finally with CAS Block (Zymed,South San Francisco, CA) for 15 min to block nonspecific binding. Staining for nitrotyrosine was performed first. Sections were incubated with a 1 mg/ml solution of polyclonal rabbit anti-nitrotyrosine antibody in PBS (a gift from Dr. J. S. Beckman, Univ. of Alabama, Birmingham, AL) for one hour followed by incubations with sheep anti-rabbit $F(ab')_2$ conjugated to biotin (Dako), and horseradish peroxidase conjugated to strepavidin (Dako) for 10 minutes each. The complex was then treated with diaminobenzamide (Dako), resulting in a brown precipitate. To control for nonspecific binding of the anti-nitrotyrosine antibody, an additional negative control consisting of a section incubated with anti-nitrotyrosine and a 10 mM solution of 3-nitro-L-tyrosine was analyzed in parallel with each set of samples. To visualize cells expressing inducible nitric oxide synthase, sections were incubated with polyclonal rabbit anti-murine iNOS antibody (10 µg/ml) in PBS for 2 hours, then with goat anti-rabbit IgG conjugated to biotin (Dako) for 10 minutes, and finally with alkaline phosphatase conjugated to strepavidin (Dako) for 10 minutes. Color was developed using fast red as the chromogen, resulting in a bright red precipitate at the site of antibody binding. The sections were counterstained with Mayer's hematoxylin.

Analysis of oxidative DNA damage

Analysis of 8-oxoguanine, an index of oxidative DNA damage, was performed only on spleens since sufficient DNA could not be obtained from lymph nodes of control mice. DNA was isolated according to the following protocol. Tissues were homogenized in lysis buffer (10 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% SDS 100 µg/ml RNaseA) with a Polytron homogenizer. After homogenization, proteinase K was added to a final concentration of 0.5 mg/ml and the samples were incubated overnight at 37°C. The DNA was fragmented by passing the lysate repeatedly through an 18 gauge needle, then through a 25 gauge needle, after which it was purified using a Qiagen Plasmid Maxi kit (Qiagen, Chatsworth, CA) using solutions and instructions provided for plasmid DNA isolation. DMSO (2%) was added to all the buffers used in the isolation procedure to prevent DNA

oxidation. DNA yield was determined spectrophotometrically and triplicate 50 μg aliquots were placed in siliconized quartz vials, where the DNA was dried and then hydrolyzed in 60% formic acid for 1 hour at 100°C; the formic acid was then removed by centrifugation under vacuum. Immunoaffinity columns were used to purify 8-oxoguanine as described by Ravanat, et al.,(28) but with the following modifications. Hydrolyzed DNA was resuspended in 0.5 ml PBS and applied, then reapplied, to the column 3 times. The column was then washed under gravity-induced flow successively with 2.0 ml of PBS, 2.0 ml of water and 1.0 ml of acetonitrile. Bound 8-oxoguanine was then eluted with 1.5 ml of methanol, 150 μl of N^2-methyl-8-oxoguanine solution (10 pg/μl) was added and the eluate was dried by centrifugation under vacuum. The dried residue was redissolved in 50 μl of the HPLC mobile phase prior to injection onto a HPLC-ECD system. The HPLC-ECD system consisted of a Hewlett Packard model 1050 pump, an ESA model 5100A Coulochem electrochemical detector and an ESA model 5010 analytical cell operated at 0.4V. Separation was achieved using a Supelco LC-18-DB 5 μm column under isocratic conditions. The mobile phase was 2% methanol in 50 mM ammonium acetate (pH 5.5) with a flow rate of 1 ml/min.

Results

Production of nitric oxide

Four groups of animals were used in the study: SJL mice bearing the RcsX tumor; mice bearing the RcsX tumor and also treated with the nitric oxide inhibitor NMA in their drinking water; untreated control mice; and control animals also receiving NMA. Mice were intraperitoneally injected with 10^7 RcsX lymphoma cells on the first day of the experiment and urinary nitrate, the final metabolite of nitric oxide *in vivo*, was quantified as measure of total nitric oxide production. Urinary nitrate excretion showed that nitric oxide production was strongly elevated in RcsX tumor-bearing mice, and that the increase was abolished by administration of NMA in the drinking water (Table 1). Tissues from these animals were used to investigate aspects of tissue damage related to elevated nitric oxide formation, with results described below.

Frequency of apoptosis

Macrophage-derived $NO^{\bullet}$ had previously been reported to induce apoptosis *in vitro* (17). To determine whether $NO^{\bullet}$ produced *in vivo* would act similarly, the frequency of apoptosis was estimated in cells of lymph nodes of RcsX tumor-bearing and control mice, and a significant increase ($P < 0.05$) was observed in tumor-bearing mice as compared to untreated control animals (Figure 1). Administration of NMA led to marginal inhibition of apoptosis ($P < 0.093$). It is noteworthy that numbers of apoptotic nuclei occurring in different areas of the same section were highly variable (see Figure 2a below). These results provide evidence that nitric oxide acted as a cytotoxic agent *in vivo*.

Immunohistochemistry of iNOS and Apoptosis

Close spatial relationships between $NO^{\bullet}$-producing and apoptotic cells would provide further evidence supporting the suggestion that apoptosis was stimulated by $NO^{\bullet}$ under these conditions. To study relationships between sites of $NO^{\bullet}$ production and apoptosis, a protocol permitting TUNEL and iNOS immunohistochemistry on the same tissue section revealed that apoptotic nuclei were concentrated in areas containing numerous iNOS expressing macrophages, and most apoptotic nuclei were located no more than three cell diameters away from iNOS-expressing cells (Figure 2). Apoptotic nuclei were observed not only in cells juxtaposed to macrophages producing $NO^{\bullet}$, but also within the macrophages themselves (Figure 2). These findings provide additional support for the suggestion that nitric oxide plays a role in the induction of apoptosis *in vivo*, and also indicate that under conditions of high endogenous $NO^{\bullet}$ production, both the generator cell

and its neighbors may undergo apoptosis.

Co-immunohistochemistry of iNOS and nitrotyrosine

The toxic effects of nitric oxide under these *in vivo* conditions could, potentially, be attributable to peroxynitrite ($ONOO^-$), formed by reaction of $NO^\bullet$ with superoxide anion in a diffusion-limited reaction (19, 29). Peroxynitrite can nitrate the phenolic ring of tyrosine (30) a product previously found to be associated with the inflammatory state *in vivo* (31-33). To characterize further quantitative and spatial relationships between macrophage-derived $NO^\bullet$ production and nitrotyrosine formation *in vivo*, we applied a co-immunostaining procedure for iNOS and nitrotyrosine, and detected iNOS and nitrotyrosine in cells of the spleen and lymph nodes of RcsX tumor-bearing SJL mice, but not in tissues of control animals. In tumor-bearing mice not administered NMA, nitrotyrosine staining in the spleen and lymph nodes was concentrated in regions where iNOS was also expressed (Figure 3). In the spleen, the general distribution pattern of nitrotyrosine expression closely resembled that of iNOS expression around the follicular regions. Nitrotyrosine staining did not appear to localize to specific cell types or subcellular organelles but was, rather, diffuse. The intensity of nitrotyrosine staining varied greatly among individual cells. Some nitrotyrosine staining was present in iNOS-expressing cells but the majority was found in adjacent cells. Furthermore, not all iNOS-expressing cells contained nitrotyrosine residues despite its presence in adjacent cells. Cells in tissues of RcsX tumor-bearing mice receiving 30 mM NMA in drinking water stained for iNOS but did not contain detectable levels of nitrotyrosine (data not shown). We conclude that production of $NO^\bullet$ by macrophages in lymphatic organs of RcsX tumor-bearing SJL mice led to localized formation of nitrotyrosine in both $NO^\bullet$ producing and adjacent cells. However, the formation of $NO^\bullet$ frequently occurred in the absence of nitrotyrosine staining.

Oxidative DNA damage

Nitric oxide has been reported to induce oxidative DNA damage in cell-free systems and macrophage cell lines (34, 35). To investigate whether macrophage-derived $NO^\bullet$ led to oxidative damage *in vivo*, 8-oxoguanine, a marker of oxidative DNA damage, was quantified in DNA isolated from spleens of tumor-bearing and control mice. No increase in 8-oxoguanine levels was observed: the value in DNA from control animals was 9.90 ± 1.64; in RcsX-bearing animals, 10.47 ± 7.35; and in RcsX-bearing animals receiving NMA, 8.54 ± 6.35 adducts per 10^6 bases.

Peroxynitrite has been reported to induce peroxidation of membrane lipids, leading to the formation of malondialdehyde (36). We therefore hypothesized that nitric oxide might cause DNA damage indirectly by formation of M1-deoxyguanosine adducts (3-ß-D-2'-deoxyribofuranosylpyrimido[1,2-a]purin-10(3H)-one) generated through this route (37). Dr. C. A. Rouzer and L. J. Marnett at Vanderbilt University generously analyzed DNA isolated from spleens of RcsX tumor-bearing and control SJL mice for this adduct by the previously described method (37). All samples contained less than 30 picograms of M1-deoxyguanosine, the detection limit of the analytical procedure. However, these data must be regarded as inconclusive, inasmuch as the maximum quantity of DNA that could be recovered from the spleen of control mice for analysis was 200 micrograms. The sensitivity of the analytical procedure was limited by this constraint. Taken together with the negative findings with respect to 8-oxoguanine levels, these results suggest that oxidative DNA damage does not accumulate to significant levels in the spleen of RcsX tumor-bearing mice.

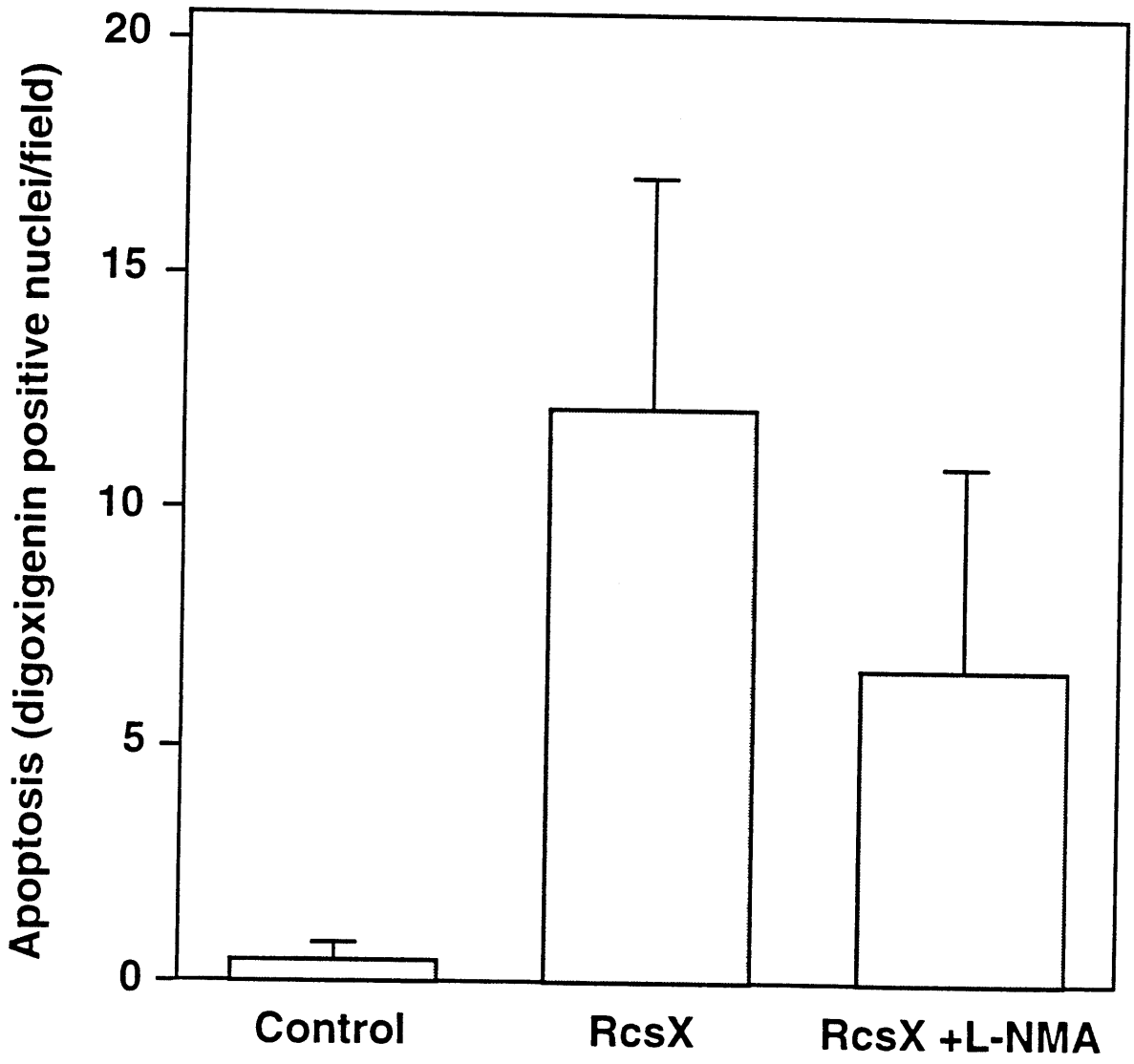
Table 1: Excretion of urinary nitrate by RcsX tumor-bearing SJL mice. Significant elevation in nitrate excretion was observed after i.p. administration of 10^7 RcsX cells. The increase was abrogated by the addition of 30 mM NMA to the drinking water of the mice.

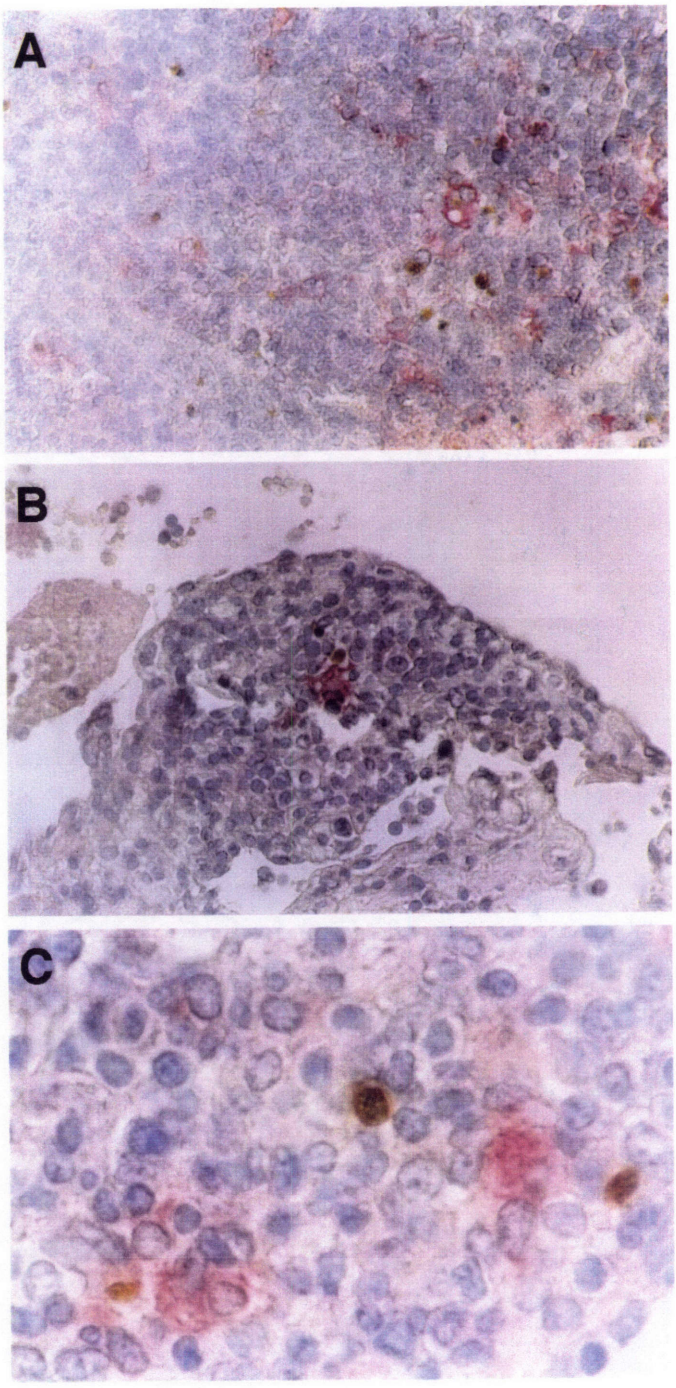
Figure 1: Apoptosis in lymph nodes of RcsX tumor-bearing SJL mice and controls. Apoptosis was analyzed in five animals per treatment group. Significant differences ($P < 0.05$) were observed between control and tumor-bearing mice. Marginal differences ($P < 0.093$) were observed between tumor-bearing mice with and without NMA.

Figure 2: Immunohistochemistry of inducible nitric oxide synthase (red) and detection of apoptosis by TUNEL (brown). All illustrations are from RcsX tumor-bearing mice: a) Spleen, 400X; b), Lymph node, 400X; c) Lymph node, 1000X.

Figure 3: Immunohistochemical analysis of inducible nitric oxide synthase and detection of nitrotyrosine. All illustrations are from RcsX tumor-bearing mice: a) Inducible nitric oxide synthase in spleen, 100X; b) Nitrotyrosine in spleen, 200X; c-d) Inducible nitric oxide synthase (red) and nitrotyrosine (brown) in lymph node, 400X and 1000X, respectively.

	day 1	day 4	day 8	day 12
RcsX treated	20.2 ± 17.5	79.1 ± 54.3	140.4 ± 42.5	724.4 ± 140.6
RcsX/NMA	5.8 ± 3.4	6.1 ± 1.8	9.0 ± 3.3	68.3 ± 41.0
NMA alone	8.6 ± 5.0	12.3 ± 6.5	13.8 ± 6.1	19.6 ± 8.3
Untreated	19.7 ± 16.3	24.1 ± 18.2	26.0 ± 13.3	29.6 ± 18.8


Apoptosis (digoxigenin positive nuclei/field)
20
15
10
5
0
Control
RcsX
RcsX +L-NMA


A
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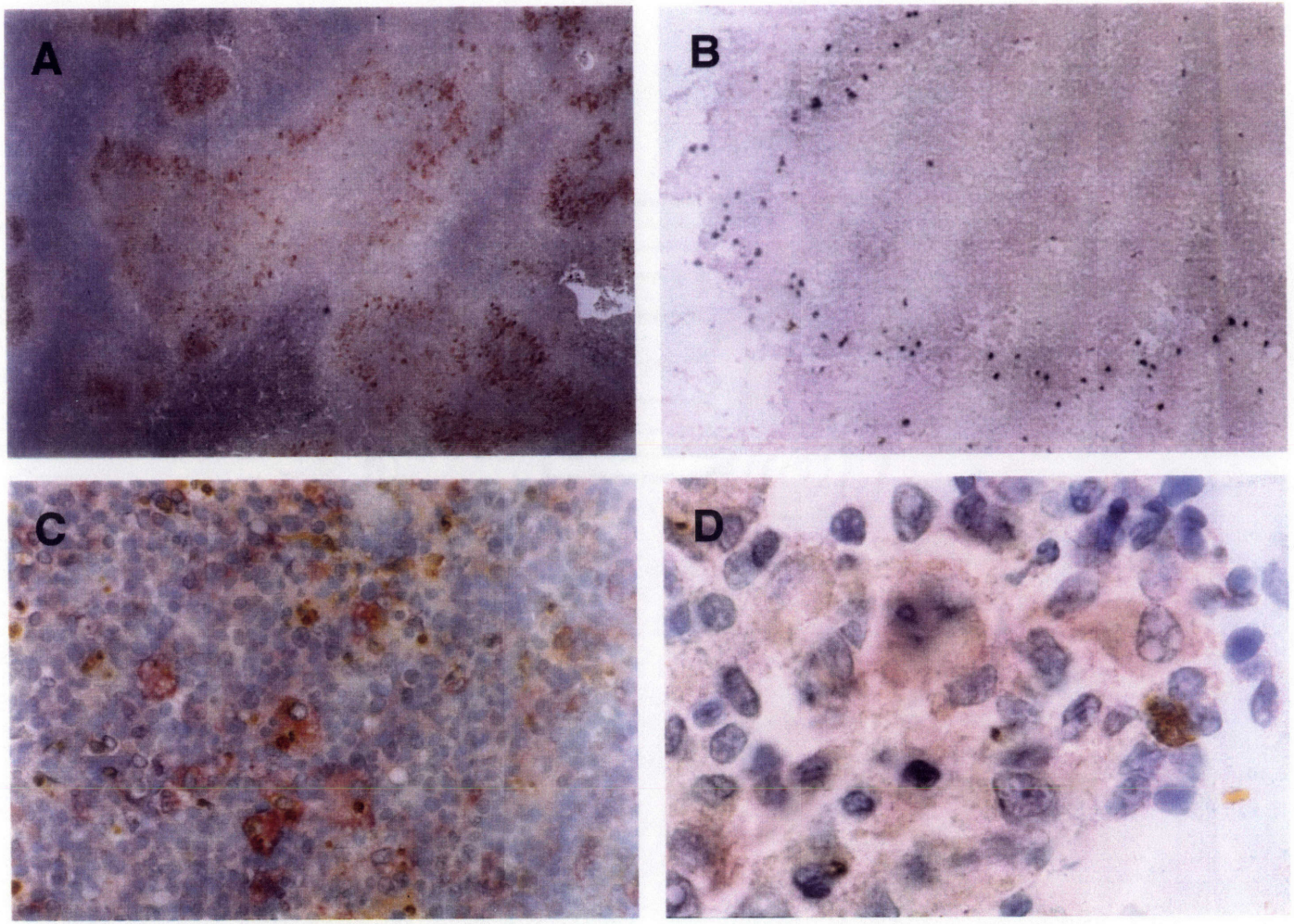
C


A
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C
D

Discussion

SJL mice are predisposed to autoimmune diseases and cannot suppress the inflammatory reaction induced by superantigen-bearing RcsX lymphoma cells (26, 38, 39). As part of the inflammatory reaction, spleen and lymph node-macrophages are activated to produce nitric oxide (24, 25), thus subjecting splenocytes to prolonged exposure to elevated levels of the radical. Complementary approaches were used to investigate toxicological consequences of this exposure. To study spatial relationships between nitric oxide formation and its effects, iNOS, apoptotic nuclei and nitrotyrosine were immunohistochemically co-localized. To quantify damage to DNA, levels of 8-oxoguanine were measured in DNA isolated from tumor-bearing mice.

The immunohistochemical results clearly demonstrate that nitric oxide formation resulted in toxicity. The number of apoptotic nuclei correlated positively with iNOS expression ($NO^{\bullet}$ production) in lymph nodes of tumor-bearing mice. Cells undergoing apoptosis were concentrated around cells expressing iNOS, were intermingled with non-apoptotic cells, and were commonly juxtaposed to non-apoptotic $NO^{\bullet}$-producing macrophages.

Several mechanisms could account for these observations. Lancaster (40) modeled the diffusion of nitric oxide and concluded that $NO^{\bullet}$ concentration would decrease by 50% for every 8 cell diameters traversed (160 μm, assuming $t_{1/2}$=4 sec.). In the spleen and lymph nodes of RcsX tumor bearing mice, nitric oxide producing cells were close enough to each other to allow substantial overlap of their diffusion spheres (Figures 2a, 3a). Thus, in lymph nodes and areas of high $NO^{\bullet}$ production in the spleen, a field of nitric oxide can be hypothesized to exist, within which the local concentration of nitric oxide would depend on proximity to multiple, not single, $NO^{\bullet}$-producing macrophages. Responses to the $NO^{\bullet}$ field by individual cells are likely to depend on cell type. For example, exogenously delivered $NO^{\bullet}$ has been shown to cause apoptosis in mouse thymocytes (41), pancreatic beta cells (42, 43), and chondrocytes (44), but, in contrast, to decrease apoptosis in splenic B lymphocytes (45). The ability of nitric oxide to induce apoptosis may also be dependent on synergism with other macrophage derived products such as H_2O_2 and TNFα (46-48), and appears almost certain to be affected by reaction with superoxide anion ($O_2^{\bullet-}$).

Superoxide reacts with nitric oxide with rapid, diffusion-limited kinetics, to form peroxynitrite ($ONOO^-$). Under physiological conditions, superoxide has a much shorter half life than nitric oxide (less than 50 milliseconds) and a free diffusion path half life of 2

μm (49). Thus, the diffusion of NO• into areas of high $O_2^{\bullet-}$ production would result in quantitative and localized production of ONOO- Peroxynitrite is a powerful one- and two-electron donor capable of oxidizing and nitrating target molecules (19, 50), and is lethal to cells *in vitro* (51) and *in vivo* (52). Strong evidence for the toxicity of peroxynitrite *in vivo* comes from animal experiments in which tissue damage induced by immune reactions and reperfusion injury were attenuated either by inhibition of NO• production or by reduction of O_2^- levels by superoxide dismutase (22, 23).

A hypothesis to explain the toxic potential of nitric oxide and peroxynitrite has recently been proposed. Poly(adenosine 5'-diphosphoribose) polymerase (PARP) is a nuclear enzyme which, when activated by DNA strand breaks, adds up to 100 adenosine 5'-diphosphoribose (ADP-ribose) residues to nuclear proteins consuming ß-nicotinamide and ATP in the process. Excessive activation of PARP can lead to depletion of ß-nicotinamide-ADP and ATP. Snyder and co-workers observed that PARP is activated by NO•, and that addition of PARP inhibitors prevents NO•-induced neurotoxicity (53). Heller *et al.* further demonstrated that NO•-induced toxicity in pancreatic islet cells is suppressed in mice carrying an inactive PARP gene (54). Further evidence in support of a connection between strand breaks, PARP and apoptosis was provided by Szabo and coworkers who demonstrated that in macrophages and smooth muscle cells, macrophage-derived peroxynitrite induced DNA strand breaks which led to activation of PARP, depletion of intracellular NAD^+ and ATP, decrease in mitochondrial respiration, and cell death (55, 56).

In light of the toxicity of peroxynitrite, the apparent resistance of macrophages to apoptosis observed in our experiments was surprising. Tamir *et al.* calculated the average rate of NO• production by activated RAW 264.7 macrophages to be between $5x10^3$ and $5x10^4$ molecules/cell-sec. (13). Lewis and Dean used the ratio of nitrate to nitrite in activated RAW 264.7 macrophages to approximate superoxide production rate to be half that on nitric oxide (29). Our results would therefore be consistent with the possibility that activation of macrophages may also induce protective mechanisms that enable it to withstand exposure to peroxynitrite, perhaps a system analogous to the soxRS regulon of *E. coli* (57).

Our results together with previously reported observations suggest that in the spleen and lymph nodes of RcsX tumor-bearing SJL mice, fields of high nitric NO• concentration are formed, within which NO• appears to potentiate apoptotic effects of other factors rather

than directly inducing cytotoxicity in cells positioned at the top of its concentration gradient.

Under physiological conditions, nitrotyrosine is formed exclusively from peroxynitrite (58, 59) and has been identified in tissues of patients with chronic inflammatory conditions such as *H. pylori* infected stomach (60), synovial fluid from rheumatoid patients (59), arterial tissue from atherosclerotic patients (61) and lung from patients with acute lung injury (33). Nitrotyrosine was also detected in rat skeletal muscle during aging (62) and spontaneous myositis in SJL mice (our previous results, data not shown). In all these cases, nitrotyrosine was regarded to be a biomarker of inflammatory damage. The objectives of the present work were to determine whether nitrotyrosine formed in the spleen and lymph nodes of RcsX-tumor bearing SJL mice, and, if so, to elucidate relationships between iNOS production and nitrotyrosine accumulation. Results show that nitrotyrosine formation was highly dependent on nitric oxide production, and that spatial relationships between iNOS expression and nitrotyrosine staining were similar to those between iNOS and apoptosis. Nitrotyrosine-containing cells were intermingled with undamaged cells, including iNOS-expressing macrophages. The observation that nitrotyrosine accumulation occurred in islands within the nitric oxide production field, but not necessarily within $NO^{\bullet}$-producing macrophages or their immediate neighbors, reinforces the suggestion that $NO^{\bullet}$ potentiates localized damage by formation of peroxynitrite through reaction with superoxide rather than causing cytotoxicity directly.

Macrophage-induced, $NO^{\bullet}$-dependent oxidative DNA damage has been reported to occur in cell culture systems (34). Experiments *in vitro* have demonstrated that treatment with activated macrophages as well as with chemically synthesized peroxynitrite led to the formation of oxidized DNA bases in exposed cells (13, 21, 35). The high level of nitric oxide production, the presence of nitrotyrosine and the increase in apoptosis observed in our experiments, suggested that oxidative DNA damage might occur in the lymphatic tissues of RcsX tumor-bearing mice. However, we found no increase in the level of 8-oxoguanine, and preliminary data suggest that levels of M1-deoxyguanosine were also not increased in spleen DNA of these animals. This finding could be attributable to efficient repair of these adducts or by the failure of $NO^{\bullet}$ to induce the adducts under the conditions of the experiment. An additional possibility is that $NO^{\bullet}$ did indeed induce oxidative DNA adducts that were highly localized in individual cells within or near centers of $O_2^{\cdot-}$ production, but due to the high natural background of 8-oxoguanine, these could not be detected in DNA extracted from tissues. This possibility would be consistent with our

observation that, despite its detection in individual cells by immunohistochemistry, increased levels of nitrotyrosine could not be detected on a western blot of soluble spleen proteins (data not shown).

Collectively, currently available information provides a basis for a tentative model for the role of nitric oxide in inflammation, in which infiltration of macrophages into inflamed tissue results in localized damage to specific target cells while the remainder of the tissue is less affected. Therefore, the significance of nitric oxide in inflammation and carcinogenesis may be underestimated by analysis of damage to macromolecules extracted from entire organs. Future studies will be needed to identify cells targeted by nitric oxide *in situ* and to elucidate $NO^{\bullet}$-induced molecular damage in these cells.

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4. Mutagenesis associated with nitric oxide production in transgenic SJL mice

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Abstract

We recently reported development of an experimental model for the study of nitric oxide toxicology *in vivo*. SJL mice were injected with superantigen-bearing RcsX (pre-B-cell lymphoma) cells, which migrated to the spleen and lymph nodes, where their rapid growth induced activation of macrophages to produce large amounts of nitric oxide over a period of several weeks. In the experiments described here, we used this model to investigate mutagenesis in splenocytes exposed to nitric oxide during RcsX cell growth. Transgenic mice were produced by crossbreeding animals of the pUR288 transgenic C57Bl/6 and SJL strains. RcsX cells were injected into F1 mice and nitric oxide production was confirmed by quantification of urinary nitrate, the ultimate metabolite of nitric oxide. Mutant frequency in the lacZ gene of the pUR288 plasmid was determined in DNA isolated from spleen (target) and kidney (non-target) tissues. A significant elevation in mutant frequency was found in spleen, but not in kidney of tumor-bearing mice. Furthermore, increases in mutant frequency in spleen as well as nitric oxide production were abrogated by administration of N-methylarginine, a nitric oxide inhibitor, to mice following injection of RcsX cells. These results indicate that nitric oxide had mutagenic activity in RcsX tumor-bearing mice and thus support a possible role for its involvement in the carcinogenic process.

Introduction

Elevation of risk for certain cancers by persistent inflammation has been suggested by extensive observational data (1, 2), but a causal relationship has yet to be established for any cancer. Central to proposed mechanisms through which inflammation may contribute to carcinogenesis is the migration of inflammatory cells to sites of infection or injury, accompanied by release of reactive free radicals. The radicals, in turn, are hypothesized to deregulate cellular processes, increase cell turnover and cause mutations that together facilitate the carcinogenic process (3, 4). Recent data have shown that the free radical nitric oxide ($NO^{\bullet}$) is present in inflammatory conditions associated with malignancies (5, 6), while studies in several *in vitro* experimental systems demonstrated that it is capable of inducing cellular damage and mutations (reviewed in (2, 7-9).

In order to assess *in vivo* toxicological responses to excess $NO^{\bullet}$ production in intact animals, an experimental model utilizing the SJL mouse was developed (10, 11). In this model, mice were injected with superantigen-bearing RcsX cells. The superantigen is known to stimulate host Vß16+ T cells (12) to secrete cytokines which, in turn, support the growth of RcsX tumor cells (13). In the course of this immune response, iNOS expression was induced in macrophages located in spleen and lymph nodes, resulting in a 50-fold increase in $NO^{\bullet}$ production 14 days after tumor cell injection (11).

The experiments described here were designed to investigate mutagenesis in splenocytes exposed to nitric oxide produced by splenic macrophages in response to RcsX cell growth. The target gene used was the lacZ gene of the pUR288 plasmid (14, 15), which was introduced by breeding C57B/6#60 (pUR288 transgenic mice) with SJL mice. The spleen and lymph nodes of RcsX lymphoma-bearing C57B/6 x SJL F1 (designated SJL#60) mice comprised an environment in which splenocytes were subjected to macrophage-derived nitric oxide over a prolonged period of time. A significant increase in mutant frequency was observed in the lacZ transgene recovered from DNA of spleen, but not kidneys, of tumor-bearing SJL#60 mice. The increase was prevented by administration of the nitric oxide synthase inhibitor, N^{G}-methyl-L-arginine acetate (NMA). This report demonstrates mutagenicity associated with endogenously produced nitric oxide and suggests a potential mechanism for its involvement in the carcinogenic process.

MATERIALS AND METHODS

Animals

Ten male SJL mice (Jackson labs, Bar Harbor, ME) were bred with ten female C57BL/6#60 transgenic mice homozygous for the pUR288 plasmid (kindly provided by J. Vijg, Beth Israel Hospital, Boston, MA). Southern blots were performed on DNA from male F1 offsprings to confirm that the transgene was uniformly inherited. Twelve- to fourteen-week old female C57BL/6#60 x SJL F1 mice were fed a low nitrate control diet (AYN-76A, Bioserve, Frenchtown, NJ) for three weeks to minimize the background rate of nitrate excretion. Animals were then weighed, placed in pairs in metabolic cages and their drinking water was replaced with either a 30 mM solution of the nitric oxide synthase inhibitor N^G-methyl-L-arginine acetate (NMA) (Chem Biochem Research, Salt Lake City, UT) or 30 mM ammonium acetate (Sigma Chemical Co., St. Louis, MO). Two days later, six mice receiving NMA and six mice receiving ammonium acetate were each injected intravenously with 0.2 ml PBS containing 10^7 cells of the RcsX line (kindly provided by N. Ponzio, State University of New Jersey Medical Center, Newark NJ) isolated from lymph nodes of SJL mice bearing actively growing tumors. Urine was collected on alternate days into tubes containing 0.5 ml 0.5 M NaOH to inhibit bacterial growth. As a measure of endogenous production of nitric oxide, total urinary nitrate concentration was determined as previously described (16) and normalized to body weight. Animals were killed by CO_2 asphyxiation 20 days after injection of RcsX cells; spleen, liver and kidneys were removed from each animal and immediately frozen in liquid nitrogen.

DNA Isolation

Tissues were homogenized by Polytron sonication in lysis buffer (10 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% SDS, 100 µg/ml ribonuclease A). After homogenization, proteinase K was added to a final concentration of 0.5 mg/ml, and the samples were incubated overnight at 37°C. Samples were extracted once with phenol/chloroform/isoamyl alcohol (10:10:1 v/v), and twice with chloroform. DNA was precipitated in cold absolute ethanol, spooled onto a glass rod and washed with 5 ml cold ethanol. Purified DNA was air dried for 10 minutes and solubilized in 0.5 ml TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

Plasmid Rescue and Mutation Assay

The mutation assay was performed essentially as described by Dolle et al. (15),

based on the following general strategy. The pUR288 transgenic plasmid contains the *E. coli* pBR322 origin of replication together with lacZ and ampicillin resistance genes. The plasmid is integrated into the genome of C57BL/6#60 transgenic mice in tandem arrays containing 40 copies of the lacZ gene. In these experiments, the plasmid was isolated by digestion of tissue DNA with HindIII, followed by incubation with magnetic beads coated with lacI protein, which binds the lacZ promoter, discarding the unbound portion and subsequently adding IPTG to release the plasmid from the beads. The isolated plasmid was circularized by ligation and transformed by electroporation into the indicator bacterial strain *E. coli C (Δlac, galE).* Plating efficiency was determined by plating 0.1% of the transformed bacteria on medium containing ampicillin and X-gal; mutants were identified and enumerated by plating the remainder of the bacteria on medium containing ampicillin and phenyl-ß-D-galactopyranoside. In the presence of active ß-galactosidase, phenyl-ß-D-galactopyranoside is converted to galactose. The first two genes of the galactose operon convert galactose to UDP-galactose. Mutational inactivation of galE, the third gene in the operon, blocks conversion of UDP-galactose to UDP-glucose, resulting in accumulation of the toxic intermediate UDP-galactose in bacteria expressing functional lacZ (14, 17). Thus, bacteria harboring wild type lacZ plasmids grow in the presence of ampicillin alone (plating efficiency), but fail to grow in the presence of phenyl-ß-D-galactopyranoside. In contrast, bacteria containing mutant lacZ plasmids grow under both conditions. Mutant frequency is therefore expressed as the ratio of phenyl-ß-D-galactopyranoside resistant colonies to ampicillin resistant colonies.

Isolation and Characterization of Mutant Plasmids

Total numbers of mutants isolated from specific tissue DNA samples varied from 7 to 139 (see Table 1). Whenever possible (the majority of instances), 24 independent mutant bacterial clones were selected from each mouse for characterization; otherwise, all mutants isolated were characterized. Mutant colonies were transferred from selective medium into 2 ml tubes containing 500 µl TB-media (12 g/L Bacto-tryptone, 24 g/L Bacto-yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, 25 µg/ml kanamycin and 75 µg/ml ampicillin) and incubated overnight at 37°C.

To identify lacZ mutants containing large deletions or insertions, crude DNA preparations were isolated from bacteria in 400 µl of each culture and subjected to electrophoresis on a 1% agarose gel according to the method of Liu and Mishra (18). Bacteria containing DNA with gel mobility of the wild type plasmid were cultured in 6 ml

TB medium overnight at 37°C, after which plasmid DNA was isolated and purified using the Perfect Prep kit (5 Prime, 3 Prime, Boulder, CO). Sequences of the lacZ gene in mutant plasmids were then determined by automated cycle sequencing (Molecular Genetics Facility, University of Georgia, Athens, GA) using the following primers: LacZ170: 5'-ttgtgtggaatt gtgagagcgg-3', LacZ781: 5'-tgctgcgttg gagtgacggc-3', LacZ1382: 5'-ctgttcgcatt atccgaacc-3', LacZ1984: 5'-gcggtgattt tggcgatacg-3', LacZ2581: 5'-cgctggataa cgacattggc-3'. Mutations were characterized by comparison of wild type and mutant sequences with the Sequencher program (Gene Codes Corporation, Ann Arbor, MI).

Statistics

Chi square analysis revealed no excess variability among experiments performed on different days with respect to observed mutation frequency. Similarly, no excess variability was evident with respect to mutation frequency in DNA from kidneys of individual mice. Therefore, statistical analysis of mutation frequencies in kidney DNA was carried out on unpaired samples by the one-tailed Student's *t* test. Conversely, in the case of spleen DNA, excess mouse-to-mouse variability and unequal variances were found to exist in mutation frequencies in different treatment groups; therefore, mutant frequencies of spleen DNA samples were compared using the two-sample *t* test for independent samples with unequal variances (Satterwaite's method) as suggested by Piegorsch et al. (19) and described by Rosner (20).

RESULTS

Tumor Cell Growth and Nitric Oxide Production

The objective of this study was to adapt the pUR288 transgenic mutant detection system to the RcsX tumor-bearing mouse model, in order to assess the mutagenicity of endogenously produced nitric oxide. We first attempted to generate a pUR288 transgenic mouse in the SJL background by the conventional plasmid microinjection method, but were unsuccessful due to poor reproductive performance of SJL mice. Therefore, it was necessary to produce transgenic animals by crossbreeding animals of the C57BL/6#60 strain with SJL mice (F1 offspring designated as SJL#60). The two strains were known to be antigenically compatible, and C57BL/6#60 animals had previously been shown to support the growth of SJL-derived RcsX cells (N. Ponzio, UNJMC, Newark NJ, personal communication). Preliminary experiments in our laboratory confirmed the growth of RcsX cells, and also showed that iNOS was expressed in the spleen of RcsX tumor-bearing SJL#60 mice (data not shown).

Groups of eight transgenic mice treated as follows were used in this study: mice bearing the RcsX tumor; mice bearing the RcsX tumor and also treated with the nitric oxide inhibitor NMA in their drinking water; untreated control mice; and control animals also administered NMA. Mice were each injected intravenously with 10^7 RcsX lymphoma cells on the first day of the experiment and urinary nitrate, the final metabolite of nitric oxide *in vivo*, was quantified to confirm nitric oxide production. Urinary nitrate excretion showed that nitric oxide production was strongly elevated in RcsX tumor-bearing mice ($P < 0.05$ by day 6), and that the increase was abolished by administration of NMA in the drinking water (Fig. 1).

Administration of NMA alone did not affect the body weight of the animals, and no differences were observed in the weights or gross appearance of organs from mice receiving NMA and those from mice receiving ammonium acetate in the drinking water. Spleens from RcsX-injected mice receiving ammonium acetate weighed 0.34 ± 0.09 grams and those from treated mice receiving NMA weighed 0.31 ± 0.07 grams; peripheral lymph nodes from animals receiving the same treatments weighed 0.083 ± 0.30 and 0.078 ± 0.19 grams, respectively.

Mutant Frequencies

Mutant frequency was determined in eight spleens (target organ) and four kidneys (nontarget organ) isolated from RcsX tumor-bearing and control mice, and results are summarized in Tables 1 and 2. In spleens (Fig. 2), a highly significant difference was

observed between mutant frequencies in RcsX tumor-bearing mice and those of all other treatment groups (compared to control mice: $t = 4.18$, $df = 10$, $P < 0.005$; compared to RcsX + NMA mice: 2.814, $df = 10$, $P < 0.010$; and compared to control + NMA mice: $t = 3.539$, $df = 11$, $P < 0.005$). A significant difference was also observed between the RcsX + NMA and control groups ($t = 2.097$, $df = 13$ $P < 0.050$), but not between RcsX + NMA and control + NMA groups ($t = 1.187$, $df = 13$, $P < 0.250$). No differences were observed between mutant frequencies observed in kidneys of animals of any of the treatment groups. We therefore conclude that growth of the RcsX tumor in SJL#60 mice was mutagenic in host splenocytes and propose that nitric oxide and/or other reactive species produced in response to tumor growth was responsible for the observed mutagenicity.

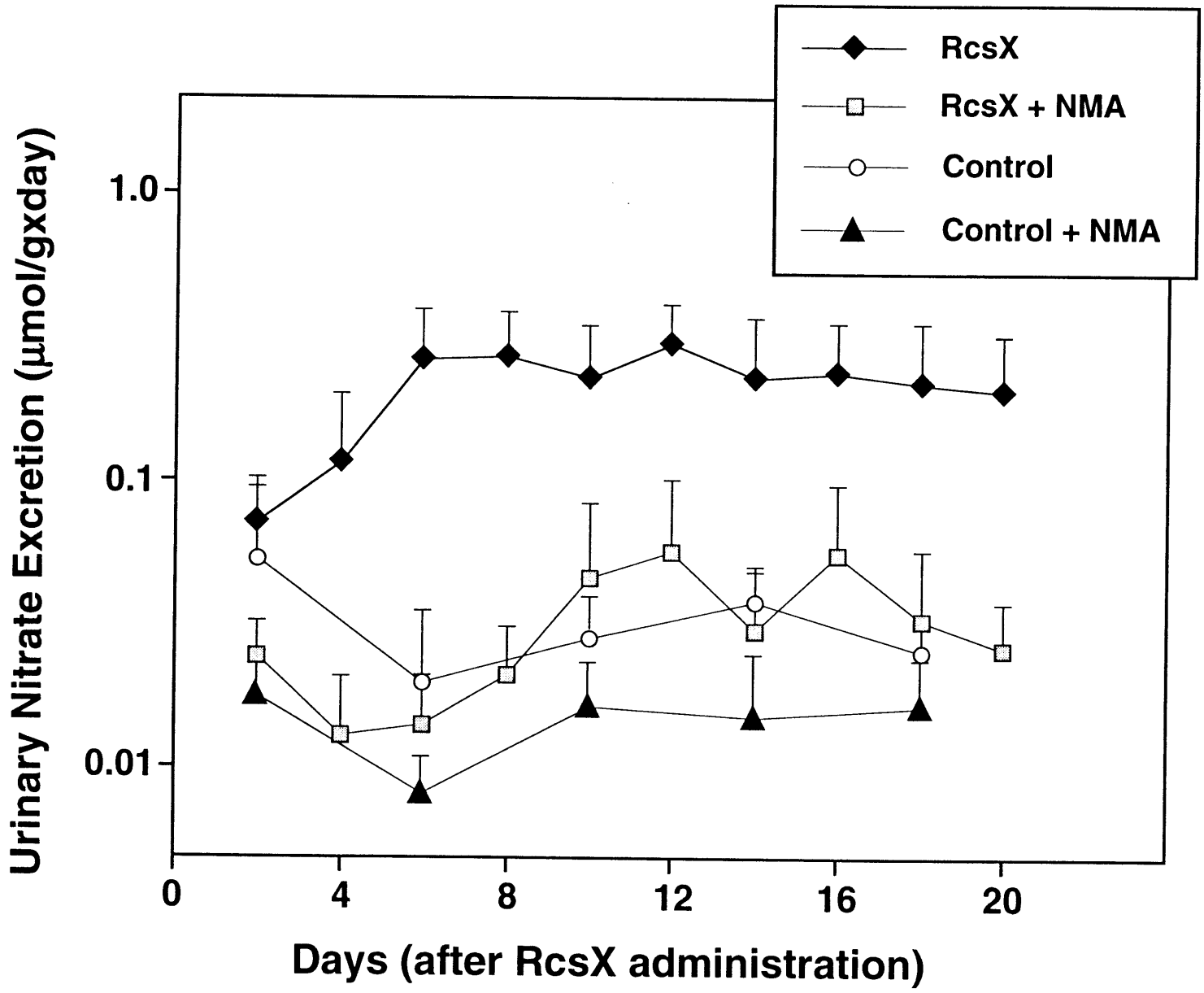
Characterization of Spontaneous and Induced Mutants

Activated macrophage-derived nitric oxide has previously been reported to induce DNA strand breaks (7, 21). We hypothesized that misrepaired strand breaks may lead to large deletions in the lacZ transgene in RcsX tumor-bearing mice. To test this hypothesis, mutant plasmids isolated from control and RcsX tumor-bearing mice were sized by migration through agarose gel. An increase in the fraction of mutants with a change in the apparent molecular weight was observed [control: 67/132 (51%), RcsX treated: 94/161 (58%)] but the increase was not statistically significant.

To further characterize the mutations induced by nitric oxide, plasmids which migrated through the agarose gel with the same apparent molecular weight as wild type plasmids were sequenced. Twenty eight spontaneous and twenty four RcsX-induced independent mutants were identified, and the results are reported in Tables 3 and 4. No difference in the patterns of mutations was observed between the two treatment groups (Table 5) although the ratio of G to A transition to G to T transversion was higher in the RcsX-induced group than controls (8/4 and 7/6, respectively).

Fig. 1) Nitrate excretion by RcsX bearing SJL#60 mice and controls. Twelve SJL mice were injected i.v. on day 1 with 10^7 RcsX cells. Eight RcsX-bearing and eight control mice received 30 mM NMA in drinking water starting on day 2. All mice were killed on day 20.

Fig. 2) LacZ mutant frequency in the spleen of RcsX tumor-bearing SJL#60 mice.


RcsX
RcsX + NMA
Control
Control + NMA
Urinary Nitrate Excretion (µmol/gxday)
1.0
0.1
0.01
0
4
8
12
16
20
Days (after RcsX administration)

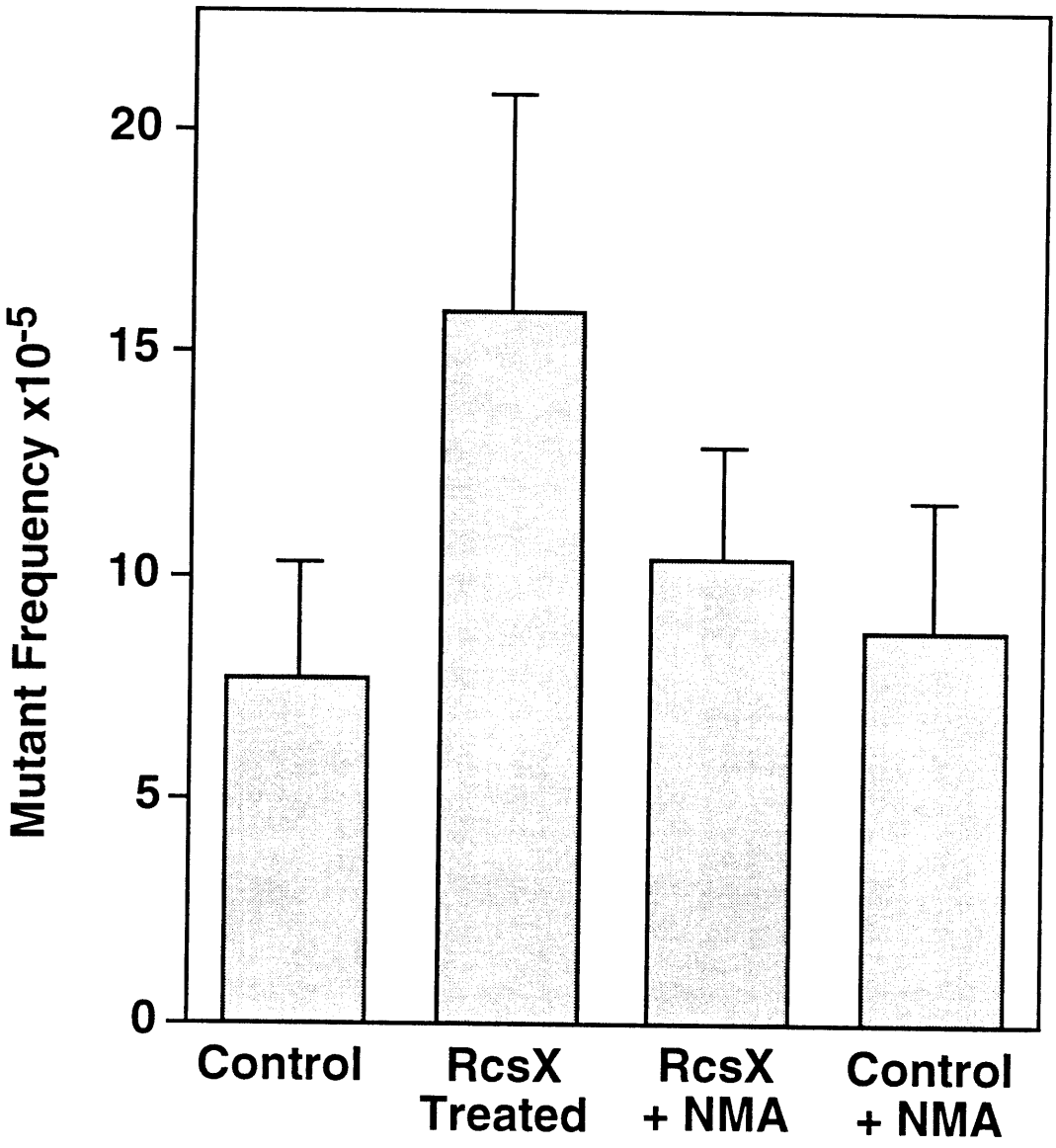

Mutant Frequency x10-5
20
15
10
5
0
Control
RcsX Treated
RcsX + NMA
Control + NMA

Table 4.1: LacZ mutant frequencies in kidneys of RcsX tumor bearing and control mice

Treatment	Animal code	No. of colonies ($x10^5$)	No. of mutants	Mutant frequency ($x10^{-5}$)
Control	307	2.90	30	10.3
	310	4.60	30	6.5
	303	2.04	21	10.3
	304	1.72	16	9.3
				9.1 ± 1.8
RcsX	101	3.70	36	9.7
	109	4.12	39	9.5
	104	2.96	24	8.1
	111	1.28	16	1.2
				8.7 ± 1.6
RcsX + NMA	208	3.80	56	14.7
	206	8.64	59	6.8
	201	1.68	12	7.1
	207	3.32	20	6.0
				8.7 ± 4.1
Control + NMA	401	5.52	41	7.4
	407	3.00	30	10.0
	405	1.64	17	10.4
	403	2.52	18	7.1
				8.7 ± 1.7

Table 4.2: LacZ mutant frequencies in spleens of RcsX tumor-bearing and control mice

Treatment	Animal code	No. of colonies ($x10^5$)	No. of mutants	Mutant frequency ($x10^{-5}$)
Control	303	5.60	67	12.0
	305	1.28	7	5.5
	306	1.68	6	3.6
	307	6.00	54	9.0
	301	5.12	50	9.8
	304	2.62	20	7.6
	309	2.16	16	7.4
	310	9.80	70	7.1
				7.7 ± 2.6
RcsX	103	8.80	139	15.8
	105	4.04	65	16.1
	106	2.72	25	9.2
	111	1.92	25	13.0
	104	3.56	43	12.1
	109	3.68	69	18.8
	101	4.86	123	25.3
	112	3.56	60	16.9
				15.9 ± 4.9
RcsX + NMA	203	8.52	99	11.6
	207	10.56	106	10.0
	206	2.48	18	7.3
	204	12.08	84	6.9
	201	9.40	100	10.6
	208	6.48	82	12.7
	205	3.60	52	14.4
	210	6.72	66	9.8
				10.4 ± 2.5
Control + NMA	406	7.96	64	8.0
	402	9.52	74	7.8
	401	2.40	13	5.4
	404	5.32	37	6.9
	403	4.08	36	8.8
	405	3.24	25	7.7
	407	2.16	24	11.1
	409	1.70	25	14.8
				8.8 ± 2.9

Table 4.3: Spontaneous mutants in SJL#60 spleen

Mouse ID	Position	Mutation	Sequence	AA change
309	266	G:C to A:T	ACTG**G**GAAA	Trp to stop
309	285	C:G to T:A	TACC**C**AACT	Gln to stop
303	318-340	multiple deletion		frameshift
303	409	A:T insertion	TTTC_CGGC	frameshift
309	417	G:C to T:A	ACCA**G**AAGC	Glu to stop
305	441	G:C to T:A	GCTG**G**AGTG	Glu to stop
309	630	C:G to T:A	AGGC**C**AGAC	Gln to stop
310	642	A:T to C:G	AATT**A**TTTT	Ile to Leu
310	686	G:C to T:A	ACGG**G**CGCT	Gly silent
306	886	G:C to A:T	ATCA**G**CGAT	Ser to Asn
305	1128	G:C to T:A	CGCC**G**AAAT	Glu to stop
310	1230	G:C to T:A	GATT**G**AAAA	Glu to Lys
307	1340	G:C to C:G	TGCA**G**GATA	Gln to His
301	1705	C:G to T:A	GCCA**C**CGAT	Thr to Ile
307	1732	2 bp deletion	GCGC**GC**GTGG	frameshift
301	1749	C:G to T:A	CCAG**C**CCTT	Pro to ser
305	1810	G:C to C:G	ACGC**G**CCCG	Arg to Pro
303	1849	A:T to T:A	GGTA**A**CAGT	Asn to Ile
307	1895	1 bp deletion	AGTA**T**CCCC	frameshift
305	1900+	multiple deletion		frameshift
309	1905	A:T to C:G	TTAC**A**GGGC	Gln to Pro
306	1940	G:C to C:G	AGTC**G**CTGA	Ser silent
310	1951	A:T to T:A	AAAT**A**TGAT	Tyr to Phe
307	1966	G:C to T:A	AACG**G**CAAC	Gly to Val
310	2020	T:A to C:G	CAGT**T**CTGT	Phe to Ser
310	2087	G:C to C:G	ACCA**G**CAGC	Gln to His
303	2110	T:A to G:C	CGTT**T**ATCC	Leu to stop
305	2166	G:C to A:T	TAAC**G**AGCT	Glu to Lys

Table 4.4: RcsX induced mutants in SJL#60 spleen

Mouse ID	Position	Mutation	Sequence	AA change
106	250	T:A to G:C	GTTTTACAC	Leu to stop
101	265	G:C to A:T	GACTGGGAA	Trp to stop
106	509	G:C to A:T	ATGCGCCCA	Ala to Arg
103	512	2 bp deletion	GCGCGCCCAT	frameshift
106	675	GG to AA	TCTGTGGTG	Trp to stop
111	1145	T:A to G:C	TCTATCGTG	Tyr to stop
106	1158	G:C to T:A	GGTTGAACT	Glu to stop
111	1194	G:C to T:A	AGCAGAAGC	Glu to stop
111	1214	C:G to T:A	GTTTCCGCG	Phe silent
112	1272	C:G to T:A	GATTCGAGG	Arg to stop
106	1371	A:T tp G:C	CTTTAACGC	Asn to Asp
111	1387	C:G to T:A	TGTTCGCAT	Ser to Leu
106	1411	G:C to A:T	CTGTGGTAC	Trp to Stop
105	1524	A:T to T:A	GGCGATGAG	Met to Trp
105	1570	1bp deletion	CACCCGAGT	frameshift
103	1784	1bp deletion	AAAAAATGGC	frameshift
111	1784	1bp deletion	AAAAAATGGC	frameshift
111	1828	A:T to T:A	TGCGAATAC	Glu to Val
106	1893	T:A to A:T	TCAGTATCC	Tyr to Asn
103	1919	G:C to T:A	CTTCGTCTG	Val to Leu
101	2071	1bp insertion	CTGA_CGGA	frameshift
101	2118	C:G to T:A	CGGGCAAAC	Gln to stop
101	2130	G:C to T:A	CGAAGTGAC	Val to Leu
112	2482	G:C to A:T	AAGCGTTGG	Arg to Leu

Tabls 4.5: Types of mutations observed in RcsX and control treatment groups

mutation	Spontaneous	RcsX
G→A	7	8
Other transitions	2	2
G→T	6	4
Other transversions	8	5
Frameshift	5	5
Total	**28**	**24**

Discussion

When injected into immunologically compatible mice, RcsX cells localize to the spleen and lymphatic tissues of the host (22-24). In these target tissues, the superantigen presented on the surface of the RcsX cells induces a potent inflammatory response by stimulating Vß16+ T cells (12). We have previously shown by western blotting that this immune response resulted in iNOS expression in cells of the spleen and lymph nodes of host animals. Expression of iNOS was limited to a small number of cells in these lymphatic tissues, and iNOS-expressing cells were identified as macrophages by immunohistochemistry (11). We subsequently employed RcsX tumor-bearing SJL mice to assess the role of nitric oxide in inflammation-induced cellular damage, by characterizing the pattern of apoptosis and the presence of nitrotyrosine in cells of the spleen and lymph nodes. Both the number of apoptotic nuclei and the extent of nitrotyrosine staining were elevated in these tissues, and both were diminished by administration of NMA. Furthermore, immunohistochemical evidence demonstrated that nitrotyrosine was localized in cells lying in close proximity to macrophages expressing iNOS. The rate of apoptosis and extent of nitrotyrosine staining were unaffected in the kidneys of tumor-bearing animals (manuscript in preparation).

On the basis of this evidence, we hypothesized that exposure to the nitric oxide and other reactive species produced by macrophages in the course of tumor growth would constitute a mutagenic exposure for splenocytes of the host animal. In order to test this hypothesis, we utilized the pUR288 transgenic mouse, in which the lacZ gene serves as a mutational target. An experimental system based on a transgenic (as opposed to endogenous) target gene was chosen because of the wide range of tissues that can be studied and the technical facility involved in their use. The pUR288 system was specifically selected because it permits detection of deletions, which were predicted to increase in response to nitric oxide exposure.

Growth of RcsX superantigen-bearing lymphoma cells led to a ten-fold increase in nitrate excretion in SJL#60 mice. Thus, nitric oxide flux within spleen and lymph nodes (tissues in which nitric oxide producing macrophages were localized) was estimated to be substantially higher than in other tissues, since these account for less than 5% of the body mass of the animal. A statistically significant increase in mutant frequency was observed in spleen of tumor-bearing (15.9×10^{-5}) compared to control mice (7.7×10^{-5}). No increase was found in kidney (non target tissue) of tumor bearing animals. Furthermore, the increase in spleen was largely blocked by administration of NMA, a nitric oxide synthase inhibitor. Collectively, these observations support the conclusion that the increased mutation frequency was induced principally by nitric oxide. With respect to mutation frequencies observed in spleen, overdispersion and substantial differences among variances of different treatment groups existed. Similar findings have been reported in data from other transgenic mouse models (19, 26, 27) and, considering the complex nature of this model, were not surprising.

It is of interest to compare the two-fold increase in mutant frequency observed in our experiment with previously reported responses of transgenic mice to well-characterized chemical mutagens. Increased mutation frequency induced in the livers of pUR288 transgenic C57B6 mice by administration of ENU at a dose of 250 mg/kg amounted to 11.7-fold; benzo[a]pyrene at 50 mg/kg, 5.9-fold; and X-rays at 50 rads divided over 5 days, 4-fold (25). It should be noted that these dose levels were employed in order to induce maximal responses. By comparison, the increase observed in our experiments, which we propose was attributable to nitric oxide and/or other reactive chemical species, was substantial in magnitude. Several factors may have been significant determinants of the magnitude of the response, as well as the difference in potency in comparison with other mutagens studied to date. Within spleen (the target tissue), the kinetics of nitric oxide production exposed splenocytes chronically over a prolonged period, in contrast to the short-term, massive doses used in studies of other mutagens. Nitric oxide was therefore less likely to have overwhelmed cellular defenses and repair mechanisms. Further,

delivery of nitric oxide was localized and coordinated as part of a repertoire of radicals and signalling molecules produced by macrophages (28-30). Thus, relatively few potential target cells may have been exposed to a nitric oxide flux large enough to have induced mutations. Patterns of nitrotyrosine staining and apoptosis previously observed in this model support this hypothesis.

On the other hand, the increase in mutant frequency observed in these experiments was relatively large compared to reported age-dependent rates of mutation accumulation. The two fold increase in mutant fraction observed by us was approximately equivalent to the annual increases in splenic mutant frequency in the lacI phage system (3.5×10^{-5} in newborn to 15.3×10^{-5} in two year old mice (31)); the lacZ phage system (3.2×10^{-5} in newborn to 8.3×10^{-5} in one year old mice (32)); and the pUR288 plasmid system (7×10^{-5} in 6 week old mice to 12×10^{-5} in 18 month old mice (25)). The causes of spontaneous age-dependent increases in mutant frequency are unknown, but have been suggested to be attributable to endogenous production of reactive oxygen species (3). Results presented here are consistent with the postulate that endogenously produced NO• may also be a contributing factor.

The increased rate of cell replication associated with the rapid growth of RcsX cells in spleen may also have contributed to the observed increase in mutant frequency in that tissue. The importance of this factor cannot be fully evaluated on the basis of current evidence, but the antimutagenic effect of NMA suggests that it was less significant than nitric oxide as a determinant of mutation frequency. Furthermore, if cell replication was indeed involved in the process, a high fraction of sibling mutants would have been expected when mutants were sequenced. This was not the case; in a total of 34 mutants sequenced, only six were siblings.

The spontaneous mutant frequencies observed in these experiments (kidney: $9.1 \pm 1.8 \times 10^{-5}$, spleen: $7.7 \pm 2.6 \times 10^{-5}$) were somewhat higher than those previously reported by Dolle *et al.* (kidney: $5.9 \times 10^{-5} \pm 0.3$, spleen: $5.4 \times 10^{-5} \pm 0.5$). The basis for this difference is unknown, but may be related to characteristics specific to strains of mice used (SJL#60 vs C57Bl/6). The types of spontaneous mutants identified were in agreement with previous observations by Martus *et al.* and Dolle *et al.* that approximately 50% of spontaneous mutants observed in pUR288 transgenic mice involved size changes rather than base substitutions (15, 25). Base substitutions and frameshifts comprised the remaining mutations observed in our experiments, as follows: G:C to A:T transitions, 7/28 (25%); G:C to T:A transitions, 6/28 (21%); and frameshifts, 5/28 (18%). Very few base substitutions have previously been identified in spontaneous mutants of pUR288 transgenic animals, of which 3/14 (21%) were G:C to A:T transitions (M.E.T.I. Boerrigter, Boston, MA personal communication). A larger database has been accumulated regarding the spontaneous mutant pattern in mice bearing lacI and lacZ transgenes. Zhang et al. (33) report that approximately 80% of spontaneous mutants contained single or multiple base substitutions, the remainder containing frameshifts. Base pair substitutions in the liver of lacZ phage transgenic mice occurred most often in CpG islands, with G:C to A:T transitions being most common (12/27, 45%) (34). The spontaneous mutation pattern in spleen of lacI phage transgenic mice has been studied by two groups, both of whom found that base pair substitutions accounted for 80% of the mutants. In one study, the most common were G:C to T:A transversions (6/22, 27%) (33), and in the other G:C to A:T transitions (7/16, 45%) (35).

Nitric oxide has been shown to be mutagenic in a variety of experimental systems. NO• gas was mutagenic in TK6 cells (36) and caused predominantly A:T to G:C transversions in plasmids (37) and C to T transitions in a bacterial system (38). NO• donor drugs have been shown to cause predominantly G:C to A:T transversions in plasmids replicated in bacteria (39), while peroxynitrite caused strand breaks, G:T to T:A and G:C to C:G transversions (40) in the same system. Therefore, on the basis of available evidence, no pattern of mutations that can be specifically attributed to nitric oxide has yet been characterized.

The experimental system used in our studies will be useful in extending studies of mechanisms through which inflammation contributes to carcinogenesis. Transgenic C57Bl/6 x SJL F1 mice were found to respond to growth of RcsX tumor cells by production of excess amounts of nitric oxide in spleen and lymph nodes. This response was similar to that of the SJL parental strain. In SJL mice, this syndrome develops spontaneously, and in order to take advantage of that characteristic, we are currently introducing the pUR288 transgene into SJL animals by backcrossing with transgenic C57Bl/6 mice. With the SJL transgenic animals, the possible role of nitric oxide-induced mutagenesis in the spontaneous development of lymphoma can be assessed. Specifically, mutagenesis in the mesenteric lymph nodes, where the lymphoma is believed to arise, can be evaluated at early stages, and protective effects of nitric oxide synthase inhibitors can be assessed. The pUR288 transgenic SJL mouse could also be crossed to superoxide dismutase overexpressing or deficient mice in order to address the interaction of $NO^{\bullet}$ and O_2^{-}. These and other related developments are currently in progress.

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5. Conclusions

The objective of this project was to assess the carcinogenic potential of nitric oxide *in vivo*. The challenge was to develop analytical tools necessary to extend concepts derived from the study of xenobiotic carcinogens to the study of endogenous carcinogens. To accomplish this objective, three research goals were conceived and stipulated. The first goal, was to develop a model which would mimic, as closely as possible, human exposure to nitric oxide. The development of the RcsX tumor-bearing SJL mouse where NO• is produced by activated macrophages for several weeks as part of a T cell dependent immune response has accomplished that goal. The second goal of the project was to assess the molecular consequences of NO• exposure. In this work new tools were developed to study spatial relationships between NO• and its induced damage. The application of these tools to the RcsX model led to better understanding of NO•-induced apoptosis and protein nitration. The last goal was to assess the mutagenic potential of NO•. This potential was confirmed when it was found that exposure to NO• led to a two-fold increase in mutant frequency. In summary, the evidence presented here suggests that production of nitric oxide during the inflammatory process is both toxic and mutagenic and fulfills the basic criteria of a carcinogenic compound.

This project may be continued in several directions to both solidify and elaborate the current work. DNA adduct formation by RcsX bearing SJL mice needs to be further studied, especially in light of the observed mutagenicity of nitric oxide and the potential existence of nitrated DNA bases. The mutagenicity experiment was not performed on a pure SJL background. This experiment should be repeated once a pure pUR288 transgenic SJL mouse line is established. Due to the lack of an appropriate antibody, nitrotyrosine was not quantitated in these experiments. An ELISA assay should be developed for the measurement of nitrotyrosine in this model. The combination of tools developed in this work could be used to test nitric oxide inhibitory compounds in the RcsX tumor-bearing mouse model. Other mouse models of chronic inflammation, infection and irritation could also benefit from the use of these biomarkers. pUR288 transgenic SJL mice are likely, for example, to prove useful for the study of the mutagenic potential of colonic infection. In the long run, the pUR288 transgenic SJL mouse could be bred to superoxide dismutase or other transgenic mice to help dissect the role of the interaction of different radicals in the carcinogenic process.

Two observations with great potential application were made during the current work but were not explored further. First, the efficiency of lacI binding to the lacZ

promoter is phenomenal. This system can be used to rescue specific sequences from any mixture of DNA and should be considered for use as a potential tag for integrated DNA sequences. Second, in the spleen, macrophages surrounding the follicular regions were specifically activated to produce NO• in response to antigenic (superantigenic) challenge. This observation suggests a role for nitric oxide in the regulation of the immune system.

The most fundamental question which can be asked about NO• is 'can an increase in nitric oxide production induce tumor formation *de novo* ?'. This question can be addressed in the SJL mouse by following the development of spontaneous tumor formation in SJL mice and applying the biomarkers of nitric oxide exposure developed in this project (iNOS expression, moma-2+ macrophage infiltration, nitrotyrosine staining, formation of apoptotic nuclei, mutant frequency determination) at different time points during the carcinogenic process. The elevated presence of these biomarkers in the germinal centers of the mesentery lymph nodes (the source of spontaneous lymphoma) before the appearance of a tumor would be consistent with a role for nitric oxide in lymphoma development. If the results of the experiment discussed above are positive, SJL mice should receive a life long dose of nitric oxide inhibitors to assess the role of nitric oxide in the formation of the tumor.

The results presented here are a part of an emerging body of research indicating that inflammatory conditions with high nitric oxide production should be considered human risk factors, much like exposure to the sun or consumption of dietary fat. The immune system is sometimes compared to the police, patrolling the body and defending it from enemies, both external and internal. To extend this analogy, a chronic inflammatory condition is equivalent to a police state. The immune system, in its attempt to rid the body of perceived enemies, has instead become the source of the problem.